FUNGAL SUPPRESSIVE ACTIVITIES OF SELECTED RHIZOSPHERIC *STREPTOMYCES* SPP. ISOLATED FROM *HYLOCEREUS POLYRHIZUS*

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ABSTRACT

Actinomycetes, mainly *Streptomyces* spp., have been extensively studied as potential biocontrol agents against plant pathogenic fungi. This study was aimed at isolating and screening *Streptomyces* strains from rhizosphere soils of *Hylocereus polyrhizus* collected in Kuala Pilah for potential *in vitro* antifungal activity. A total of 162 putative strains of actinomycetes was isolated from moist-heat treated soil plated on starch-casein-nitrate agar, humic-acid-vitamin agar and raffinose-histidine agar. Based on the ability to produce abundant aerial mycelium, 110 strains were categorised as Streptomycete-like. Seven main groups based on aerial mycelium colour observed in this study were grey (41.4%), white (37.7%), brown (8.0%), orange (4.3%), yellow (4.3%), green (2.5%) and black (1.9%). Three pathogenic fungi, namely, *Fusarium semitactum*, *Fusarium decemcellulare* and *Fusarium oxysporum* were isolated from the diseased stem regions of *Hylocereus polyrhizus*. The actinomycetes were screened for *in vitro* antagonistic activity against the isolated pathogenic fungi. In the qualitative screening, 23 strains were able to inhibit at least one of the three pathogenic fungi. In the quantitative screening, three strains, C17, C68 and K98, showed the highest antagonistic activity (70-89%) against all the fungal pathogens. Based on phenotypic and genotypic characterisation, the three selected actinomycete strains were identified as *Streptomyces malaysiensis* (C17), *Streptomyces cavourensis* subsp. *cavourensis* (C68) and *Streptomyces sanyensis* (K98). Antifungal metabolites produced in agar cultures of the selected isolates caused folding back, stunted and bulging of the mycelium of the pathogens. The selected strains produced a range of different metabolites in International *Streptomyces* Project (ISP 2) agar medium. The compounds were identified as geldanamycin, bafilomycins (C1, B1 and D), benzoic acid, maltophilin, dihydromaltophilin, 3,5,dihydroxy-2-methyl-benzoic acid, retimycin and lagosin using HPLC-DAD-UV analysis. Phytotoxicity screening showed that spore

suspensions of strains *Streptomyces malaysiensis* and *Streptomyces cavourensis* subsp. *cavourensis* were toxic to maize seedlings at both low (1×10^6 CFU/ml) and high dosage (1×10^8 CFU/ml) treatments. Meanwhile, the spore suspensions of strain *Streptomyces sanyensis* promoted the growth of maize seedlings at both low and high dosage treatments. In the greenhouse trials, high dosage (1×10^8 CFU/ml) treatment of the *Hylocereus polyrhizus* stems with strain *Streptomyces sanyensis* spore suspension promoted the total lengths of lateral shoots and longest adventitious roots compared to sterile distilled water treated stems. The application of spore suspension resulted in formation of lateral shoots (93.3%), total length of lateral shoots (767.3%) and the length of the longest adventitious root (75.0%) from day 45 to day 90.

ABSTRAK

Aktinomiset, terutamanya spesies *Streptomyces* telah dikaji secara meluas sebagai agen kawalan biologi yang berpotensi terhadap kulat patogen tumbuhan. Projek ini dilakukan dengan tujuan pengasingan dan penyarinagan species *Streptomyces* untuk aktiviti antikulat dalam ujian dari sampel tanah rhizosfera *Hylocereus polyrhizus* di Kuala Pilah. Sejumlah 162 pencilan aktinomiset telah diasingkan dalam media kanji nitrat kasein, vitamin asid humik dan histidine- raffinose daripada tanah yang didedahkan kepada pelakuan haba-lembap. 110 pencilan tersebut telah dikategorikan sebagai *Streptomyces* berdasarkan keupayaan untuk menghasilkan miselium udara yang banyak. Tujuh kumpulan utama warna miselium udara yang diperhatikan dalam kajian ini adalah kelabu (41.4%), putih (37.7%), perang (8.0%), jingga (4.3%), kuning (4.3%), hijau (2.5%) dan hitam (1.9%). Tiga kulat patogen iaitu *Fusarium semitactum, Fusarium decemcellulare* dan *Fusarium oxysporum* telah diasingkan dari kawasan batang pokok *Hylocereus polyrhizus* yang berpenyakit. Aktinomiset telah disaringkan untuk aktiviti antagonis *in vitro* terhadap patogen kulat. Dalam panyaringan kualitatif, 23 aktinomiset dapat menghalang sekurang-kurangnya salah satu daripada tiga kulat patogen. Dalam penyaringan kuantitatif, tiga pencilan, C17, C68 dan K98, daripada 23 aktinomiset menunjukkan aktiviti antagonis tertinggi (70-89%) terhadap kesemua kulat patogen. Berdasarkan pencirian fenotip dan genotip, tiga aktinomiset terpilih telah dikenal pasti sebagai *Streptomyces malaysiensis*, *Streptomyces cavourensis* subsp. *cavourensis* dan *Streptomyces sanyensis.* Metabolit antikulat yang dihasilkan oleh aktinomiset terpilih dalam kultur agar menyebabkan miselium kulat yang diuji terlipat, terbantut dan membonjol. Aktinomiset yang terpilih menghasilkan pelbagai metabolit yang berbeza dalam media Projek Antarabangsa *Streptomyces* 2 (ISP 2). Sebatian telah dikenal pasti sebagai geldanamisin, bafilomisin (C1, B1 dan D), asid benzoik, maltofilin, dihidromaltofilin, 3,5, dihidroksi-2-metil asid benzoik, retimisin dan lagosin

dengan menggunakan analisis HPLC-DAD-UV. Penyaringan fitotoksik menunjukkan bahawa ampaian spora aktinomiset *Streptomyces malaysiensis dan Streptomyces cavourensis subsp cavourensis* adalah toksik terhadap benih jagung dalam kedua-dua rawatan dos rendah (1×10^6 CFU/ml) dan tinggi (1×10^8 CFU/ml). Sementara itu, ampaian spora aktinomiset *Streptomyces sanyensis* menggalakkan pertumbuhan anak benih jagung dalam kedua-dua rawatan dos rendah dan tinggi. Dalam ujian rumah hijau, rawatan dos tinggi (1×10^8 CFU/ml) ampaian spora *Streptomyces sanyensis* ke atas batang *Hylocereus polyrhizus* menghasilkan peningkatan dari segi jumlah panjang pucuk lateral dan panjang akar liar terpanjang apabila dibandingkan dengan rawatan kawalan. Penggunaan ampaian spora juga menunjukkan peningkatan dalam pembentukan pucuk lateral baru (93.3%), jumlah panjang pucuk lateral baru (767.3%) dan panjang akar liar terpanjang (75.0%) dari hari ke 45 hingga 90.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
±	plus-minus
°C	degree Celsius
µg	microgram
µl	microliter
µm	micrometer
1525r	1525 reverse
27f	27 forward
A.cunn. ex Benth	Author: Allan Cunningham. Ex-Author: George Bentham
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BSR	basal stem rot
CFU/ml	colony forming unit/mililiter
DAD	diode array detector
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DSM	Deutsche Sammlung Von Mikroorganismen Und Zellkulturen Gmbh (German Collection Of Microorganisms And Cell Cultures Gmbh)
e.g	exempli gratia
Et al	et alia
f.sp	formae speciales
Foc	*Fusarium oxysporum* f.sp. *cubense*
G+C	guanine plus cytosine
HCL	hydrochloric acid
HPLC	high performance liquid chromatagaphy
HSAF	heat stable antifungal factor
HVA	humic acid vitamin agar
i.e	id est
IAA	indole acetic acid
ICZ	indolocarbazole
ISP	international streptomyces project
ISR	induction of systemic resistance
MEGA	Molecular Evolutionary Genetics Analysis
$MgCl_2$	magnesium chloride
min	minute
ml	mililiter
mm	milimeter
mM	micromolar
mm^2	milimeter square
Mol	mole
NA	nutrient agar
NaOH	sodium hydroxide
NBRC	NITE Biological Resource Center

NCBI	National Center for Biotechnology Information
NRRL	Agricultural Research Service Culture Collection.
NSA	Non-sporulation agar
Penz	Penzig
PGPR	plant growth promoting rhizobacteria
pH	power of hydrogen
pv	pathogenic variants (pathovars)
RA	Retinaculum-apertum
RF	Rectiflexibilis
RHA	raffinose histidine agar
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
S	spira
SA	sporulation agar
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
SEM	scanning electron microscope
Spp	species (plural)
USM	University Sains Malaysia
UV	ultraviolet
v/v	volume/volume
var.	variety
w/v	weight/volume

CHAPTER 1

1.0 INTRODUCTION

Fungal phytopathogens cause serious problems worldwide in agriculture and food industry by destroying crops and economically important plants in the field and during storage, especially in the subtropical and tropical regions (Pohanka, 2006). In addition, many produce mycotoxins, which are harmful to humans and livestock. For example, in Asia, rice is one of the most important staple foods. However, diseases have limited rice production, affecting annual yield loss conservatively estimated at 5% (Song and Goodman, 2001). Reports have indicated more than 70 diseases on rice are caused by fungi, bacteria, viruses or nematodes. Amongst them, rice blast (*Magnaporthe grisea*), bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*) and sheath blight (*Rhizoctonia solani*) have the most devastating effect on high productivity (Ou, 1985; cited in Song and Goodman, 2001). Besides rice, Asia is also the world's leading palm oil production zone, accounting for 90% of palm oil production. The two leading countries in this zone are Malaysia and Indonesia (Durand-Gasselin *et al*., 2005). One of the main difficulties in oil palm (*Elaeis guineensis*) plantations in Asia is stem rot diseases caused by *Ganoderma boninense*, commonly known as basal stem rot (BSR). This economically important disease causes loss of between 30% and 70% by the end of a planting cycle (Arifin *et al*., 2000). In Malaysia, this disease has long existed in coastal areas, however, recent surveys have recorded typical disease incidences of 30% on 13-year-old palms in both inland and peat soils (Rao *et al*., 2003).

All over the world, *Hylocereus* spp. have been under threat from fungal, bacterial and viral diseases. Commonly known as dragon fruit in Asia, this vine climbing cactus species which originates from South America (Crane and Balerdi, 2005) was formerly introduced by the Golden Hope Plantations in Sungai Wangi Estate,

Malaysia in the late 90s (Halimi and Satar, 2007). Some of the diseases that have been reported on the white-fleshed dragon fruit (*Hylocereus undatus*) are the cactus virus X (Liou *et al*., 2001), stem rot and fruit rot in Taiwan (Wang and Lin, 2005) and Japan (Taba *et al*., 2007) caused by *Bipolaris cactivora* (Petrak) Alcorn, stem spots in Mexico caused by *Botryosphaeria dothidea* (Valencia-Botin *et al*., 2001) and anthracnose disease in Japan (Taba *et al*., 2006) and the USA (Palmateer *et al*., 2007) caused by *Collectotrichum gloeosporioides*. Yellow species of dragon fruit (*Hylocereus megalanthus* syn. *Selenicerus megalanthus*) in Brazil have also been infected by anthracnose disease caused by *Collectotrichum gloeosporioides* (Takahashi *et al*., 2008). In Malaysia, a few types of fungi have been documented as the causative agents of disease. In a preliminary study conducted in 2007 on the dragon fruit diseases in Malaysia, it was revealed that the highest number of fungal isolates associated with diseased *Hylocereus polyrhizus* was *Fusarium semitectum* (Hew *et al*., 2008; Masratul Hawa *et al*., 2008a). In addition, *Fusarium proliferatum* was also proven to cause brownish to reddish lesions on this red-fleshed dragon fruit (Masratul Hawa *et al*., 2008a). Apart from these studies, there was also a report on the bacterial soft rot disease caused by *Enterobacter cloacae* which caused yellowish to brownish soft and watery symptoms on infected stem and fruit (Masyahit *et al*., 2009a). The occurrence of anthracnose disease, caused by *Colletotrichum gloeosporioides* (*Penz*.), which resulted in the formation of reddish-brown lesions with chlorotic halo symptoms in infected stem and fruit was also reported (Masyahit *et al*., 2009b).

The current methods of controlling phytopathogens of the *Hylocereus* spp. are through the application of pesticide and fungicide. In addition, pruning of the diseased stems on a regular basis and maintaining a good agricultural practice are also key measures in minimising if not preventing the disease occurrences. Despite these, the number of farmers opting to plant other types of crops has been on the rise due to the

devastating effects of the diseases on this fruit plant. In modern agriculture, pesticide application is still a very useful and effective method to control plant diseases. Excessive usage of agrochemicals, however, subjects the environment to pollution and has detrimental effects on a host of non-target organisms, which is why the potential use of microbial antagonists based biocontrol agents as antagonist has been addressed in many reports (Shimizu *et al*., 2000). The attention in biocontrol of plant pathogens has increased considerably over the past years, partly as a response to public concerns about the use of hazardous chemical fungicides and pesticides such as methyl bromide, and also because it may provide control of diseases that cannot, or can only partially, be managed by other control strategies (Cook, 1993). In addition, the pathogens develop resistance to the fungicides which in turn requires much stronger or new chemical to counter them. This makes the process of finding new antifungal compounds more difficult and more expensive (Campbell, 1986). Compared to the usage of chemicals or pesticides, biological control of plant diseases is slow, gives few quick profits, but can be long lasting, inexpensive and harmless to life (Dhingra and Sinclair, 1995). Biocontrol of plant diseases, especially of fungal origin, has been achieved using microorganisms such as *Trichoderma* spp. (Freeman *et al*., 2004), *Pseudomonas* spp. (Ligon *et al*., 2000), *Bacillus* spp. (Cavaglieri *et al*., 2005) and *Streptomyces* spp. (Sabaratnam and Traquair, 2002).

Actinomycetes consist of a very broad phylogenetic group of Gram-positive bacteria (Thirup *et al*., 2001). Actinomycetes, mainly *Streptomycetes*, are ubiquitous and abundant in soil (Broadbent *et al*., 1971) and tend to be well distributed through the surface-soil mass like many fungi (Singh and Mehrotra, 1980). Moreover, they are efficient producers of antifungal compounds (Doumbou *et al*., 2001). Berdy (2005) reported that actinomycetes were the richest source of secondary metabolites (45%) followed by fungi, *Bacillus* and *Pseudomonas*. These were the reasons why the present

study was primarily focused on the species of *Streptomyces* as potential fungal antagonists. The bacteria from this genus are well known for their ability to produce a wide variety of fungal cell wall-degrading enzymes such as cellulose, hemicellulose, chitinase, amylase and glucanase (Yuan and Crawford, 1995). Gottlieb (1976) reported that the antibiotics produced on or near the root surfaces (rhizosphere) and inside root tissues are able to decrease the competition for scarce food reserves by killing or inhibiting fungal growth.

Several species of *Streptomyces* have been reported to have inhibitory effects on the most common soil-borne fungi like *Fusarium oxysporum* (Getha and Vikineswary, 2002), *Pythium ultimum* (Yuan and Crawford, 1995), *Verticillium* spp. (Aghighi *et al*., 2004), *Rhizoctonia solani* (Sabaratnam and Traquair, 2002) and *Gaeumannomyces graminis* (Chamberlain and Crawford, 1999). In Malaysia, Getha and Vikineswary (2002) has shown *Streptomyces* sp. strain g10, isolated from a coastal mangrove stand, as one of the several strains that demonstrated strong activity against a range of phytopathogenic fungi. Furthermore, *in vivo* biocontrol ability of the g10 strain has also been conducted against *Fusarium oxysporum* f.sp. *cubense* (*Foc*) race 4 in tissue culture-derived banana plantlets (Getha *et al*., 2005). Also, Ismet (2003) showed that a strain from the genus *Micromonospora* isolated from coastal mangrove rhizosphere soil showed strong antifungal activity against *Pyricularia oryzae* and *Ganoderma boninense*. What makes conducting this study in Malaysia even more relevant is the fact that Malaysia has been identified as one of the 17 mega biodiversity hotspots in the world by the World Conservation Monitoring Centre, an agency of the United Nations Environment Programme. This is due to the wide range of vegetation with tropical rain forests, mangrove coastlines and high altitude mountains. Therefore, this study was undertaken to investigate the potential of using actinomycetes as a biological control agent as well as to evaluate the *in vivo* potentials of actinomycetes.

The objectives of this study were to:

a) identify the fungal pathogens that cause stem rot of *Hylocereus polyrhizus* grown in Malaysia
b) isolate and screen actinomycetes for biocontrol potential against the fungal pathogens identified
c) characterise the antagonistic actinomycetes and to obtain chemical profiles of bioactive compounds using HPLC analysis
d) conduct greenhouse trials to evaluate the plant growth promoting ability of selected actinomycetes

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Actinomycetes

Among the microorganisms, actinomycetes are the largest and most important order, economically and biotechnologically (Lam, 2006), primarily due to their ability to produce an extensive array of secondary metabolites and extracellular enzymes with diverse chemical structures and biological activities (Bull *et al.*, 2000; Goodfellow *et al.*, 1997). The name "Actinomycetes" was derived from Greek word "atkis" which means ray and "mykes" which means fungus which corresponds to their ability to produce aerial mycelium and to have a fungus like-appearance (Das *et al.,* 2008). Actinomycetes are Gram-positive bacteria, with a high guanine plus cytosine (G + C) ratio in their DNA (>55mol %), which are phylogenetically related from the evidence of 16S ribosomal cataloguing and DNA: rRNA pairing studies (Goodfellow and Williams, 1983).

These organisms are prokaryotic by nature, but are considered as transition microorganism between bacteria and fungi because they may have fungal morphology during some stage in their life cycle (Goodfellow and Cross, 1984). This group consists of genera with a variety of morphologies ranging from the coccus (*Micrococcus*) and rod-coccus cycle bacteria (e.g. *Arthrobacter*), through fragmenting hyphal forms (e.g. *Nocardia*), to genera with a permanent and highly differentiated branched mycelium (*Micromonospora*, *Streptomyces* and others) (Goodfellow and Williams, 1983). Actinomycetes are the most widely distributed group of microorganisms in nature especially in soils where they exist as saprophytes (Takizawa *et al.,* 1993). Most soil-living actinomycetes belong to the genus *Streptomyces* (Lazzarini *et al.,* 2000).

Table 2.1: Hierarchic classification of the actinomycetes based on the phylogenetic analyses of the 16S rDNA/rRNA sequence data (Stackebrandt *et al.*, 1997).

Class: *Actinobacteria*; Subclass: *Actinobacteridae*; Order: *Actinomycetales*	
Suborder	**Family**
Micrococcineae	*Micrococcaceae, Brevibacteriaceae, Cellulomonadaceae, Dermabacteriaceae, Dermatophilaceae, Intrasporangiaceae, Jonesiaceae, Microbacteriaceae, Promicromonosporaceae*
Actinomycineae	*Actinomycetaceae*
Frankineae	*Frankiaceae, Acidothermaceae, Geodermatophilaceae, Microsphaeraceae, Sporichthyaceae*
Propionibacterineae	*Propionibacteriaceae, Nocardioidaceae*
Streptomycineae	*Streptomycetaceae*
Corynebacterineae	*Corynebacteriaceae, Dietziaceae, Gordoniaceae, Mycobacteriaceae, Nocardiaceae, Tsukamurellaceae*
Micromonosporineae	*Micromonosporaceae*
Streptosporangineae	*Streptosporangiaceae, Nocardiopsaceae, Thermomonosporaceae*
Pseudonocardineae	*Pseudonocardiaceae*
Glycomycineae	*Glycomycetaceae*

They are isolated from environmental samples by applying appropriate selective pressures at various stages of the dilution plate procedure (Williams *et al.,* 1984). Actinomycetes have the ability to survive in adverse environments (McBride and Ensign, 1987). This was demonstrated by Crowe *et al*. (1984) where actinomycetes was shown to be able to accumulate high endogenous concentrations of trehalose to preserve membrane integrity which is correlated to the capacity of these organisms to resist dry conditions.

Of all the actinomycetes suborder, *Streptomyces* is the genus that is cultured most abundantly (Lee and Hwang, 2002) with over 500 species isolated. Members of this genus are generally found in soil and decaying vegetation and are distinguished by their earthly odour which is caused by a volatile metabolite known as geosmin (Gust *et al.*, 2003). Another unique morphology exhibited by this genus is the formation of spores on their aerial mycelium (Waksman, 1959). Ilic *et al*. (2007) reported that *Streptomyces* are responsible for 60% of biologically active compounds such as antifungal and antibacterial compounds or plant growth-promoting substances that were developed for agricultural purposes. Some other genera (generally called rare actinomycetes), such as *Actinoplanes, Amycolatopsis, Catenuloplanes, Dactylosporangium, Kineospora, Microbispora, Micromonospora* and *Nonomuraea* are very difficult to isolate and cultivate due to their slow growth (Hayakawa, 2008). *Streptomyces* generally produces two types of mycelia, the substrate (vegetative) mycelium, and the aerial mycelium (Hopwood, 1999). The vegetative mycelia which absorbs nutrient are made-up of a dense and complex network of hyphae usually embedded in the soil or immobilised substrate. Aerial mycelium which is a reproductive agent usually grows from the surface of the vegetative mycelium once the cell culture becomes nutrient-limited. The aerial mycelium develops into spore chains as the mature stage in their life cycle (Hopwood, 1999).

2.1.1 Distribution of actinomycetes in nature

Actinomycetes are mainly found in terrestrial habitats but they are widely distributed in a variety of other habitats including compost, river mud, and lake bottoms (Alexander, 1977). They are among the most widely distributed microorganisms in nature and institute a significant component of the microbial population in most soils (Barakate *et al.,* 2002). Actinomycetes can be found in both cultivated and uncultivated soils (Goodfellow and Simpson, 1987). As of the year 2000, near half of the 10,000 antibiotics discovered were produced by *Streptomyces* that originated in the soil (Lazzarini *et al*., 2000).

Actinomycetes are also found in aquatic environments; freshwater and marine habitats (Fenical and Jensen, 2006; Pathom-aree *et al*., 2006). In aquatic habitats, these actinomycetes are able to survive in extremes of pressure, salinity and temperature due to their unique physiological and structural characteristics and produce novel secondary metabolites that are not observed in the terrestrial actinomycetes (Radajewski *et al*., 2002). Some indigenous marine actinomycetes like *Rhodococcus marinonascens* (Helmke & Weyland, 1984) and *Salinospora* spp. have been identified from aquatic samples (Mincer *et al.,* 2002; Maldonado *et al.,* 2005; Pathom-aree *et al.,* 2006). *Salinospora* represent the first taxon to be reported solely from the ocean and it has been suggested there is a worldwide distribution of these bacteria in the oceans (Mincer *et al.,* 2002; Maldonado *et al.,* 2009). *Micromonospora* are the dominant actinomycetes isolated from several samples from streams, rivers, lake mud, river sediments, beach sands, sponge and marine sediments (Rifaat, 2003; Jensen *et al*., 2005, Eccleston *et al*., 2008). A variety of actinomycetes inhabit a wide range of plants as symbionts, parasites or saprophytes and most of them belong to the genera, *Streptomyces* and *Microbispora* (Matsumoto *et al*., 1998). Endophytic actinomycetes have ability to produce a variety of bioactive metabolites including antibiotics, plant growth promoters, plant growth

inhibitors and hydrolytic cell wall-degrading enzymes such as cellulases, hemicellulases, chitinases that can apply to agricultural usages (Getha and Vikineswary, 2002; Igarashi *et al*., 2002; Taechowisan *et al*., 2003; Hasegawa *et al*., 2006).

2.1.2 Importance of actinomycetes

With the discovery of actinomycin by Waksman and Woodruff in 1940, the role of actinomycetes as potential antibiotic produces became more obvious (Waksman and Woodruff, 1942). By the end of 1980s, actinomycetes accounted for almost 70% of naturally occurring antibiotics worldwide (Okami and Hotta, 1988). Some of the significant bioactive secondary metabolites produced consist of antibiotics (Berdy, 2005; Strohl, 2004), antitumor agents (Cragg *et al*., 2005), immunosuppressive agents (Mann, 2001) and enzymes (Oldfield *et al*., 1998; Pecznska-Czoch and Mordarcki, 1988). Over the past fifty years, substantial amount of work has been conducted in the isolation of novel actinomycetes from terrestrial sources for drug discovery programs worldwide (Lam, 2006). It was estimated roughly two-thirds of natural antibiotics have been isolated from actinomycetes, and nearly 75% of them are produced by members of the genus *Streptomyces* (Newman *et al.,* 2003; Jiménez-Esquilín and Roane, 2005). Other antibiotic contributing genera such as *Saccharopolyspora, Amycolatopsis*, *Micromonospora* and *Actinoplanes* were reported to produce a lot less (Challis and Hopwood, 2003). Secondary metabolites are generally known as organic compounds that do not have direct participation in the growth, development or reproduction of the producing organism (Martin *et al.,* 2005). In actinomycetes, the secondary metabolites are excreted during the generation of aerial hyphae from the vegetative mycelium which takes place stationary growth phase (Miguelez *et al.,* 2000). This process is believed to be triggered by fermentation conditions such as the depletion of nutrients, the biosynthesis of an inducer or a decrease in growth rate. In nature, actinomycetes

produce secondary metabolites usually as defence against predators, parasites and disease or in interspecies competition and in reproductive processes (Demain and Fang, 2000). The changes in their surroundings eventually affect the type of secondary metabolites they produce.

Actinomycetes have many roles in the environment. For example, *Streptomyces* are saprophytic bacteria that are able to decompose organic matter, especially complex polymers such as lignocelluloses, starch, chitin, hemicelluloses, pectin, keratin, natural rubber and even some man-made compounds that enter the soil as contaminants (Goodfellow and Williams, 1983; Crawford *et al.,* 1993). According to Goodfellow and Williams (1983), plant rhizosphere soils are the major habitat for actinomycetes. Here, they help plant growth by disintegrating soil organic matter or fixing atmospheric nitrogen. They also produce antibiotics which are effective against fungal infections of plants (Weller and Thomashow, 1990). In the aspects of agriculture, growing demands for low-input agriculture has given rise to greater attentions in soil microorganisms which can enhance plant nutrition and health, and improve soil quality (Jeffries *et al.,* 2003). The potential of actinomycetes as biological control agents of soil-borne root diseases in crop plants has been investigated and some *Streptomyces* species, as well as a few other actinomycetes genera, have been shown to protect several different plant species against soil-borne fungal pathogens especially in glass house experiments. Some genera have also been shown to produce herbicidal and insecticidal compounds (Crawford *et al.,* 1993). Another member of this order, *Frankia*, can fix nitrogen. They have a broad host range and can form root nodule symbioses with more than 200 species of flowering plants (Mincer *et al.,* 2002).

Some actinomycetes form parasitic associations with plants. For instance, *Streptomyces scabies*, which causes ‘common scab’ in potato and sugar beet in neutral to alkaline soils (Lambert and Loria, 1989). However, plant-pathogenic actinomycetes

do not offer much threat to agriculture as much as many other plant-pathogenic bacteria and fungi (Korn-Wendisch and Kutzner, 1992). There are also many actinomycetes which form synergistic relationship with plants (Williams *et al.,* 1984). These strains are categorised as endophytes. Endophytes are defined as ‘‘bacteria or fungi, which for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease’’ (Wilson, 1995), or ‘‘those which can be extracted from inner plant parts or isolated from surface-disinfected tissues and that do not visibly harm the plant’’ (Hallmann *et al.,* 1997a). The relationship between actinomycetes and plants is beneficial to host plants through the production of phytohormones and siderophores, nitrogen fixation as well as producing antibiotics or extracellular enzymes to protect them against pathogens (Clegg and Murray, 2002). Many reports have been published on the endophytic actinomycetes that have been isolated from the tissues of healthy plants. For example, Nimnoi *et al.* (2010) reported the isolation of endophytes from the roots of *Acacia auriculiformis* A. Cunn. *ex* Benth belonging to several genera of actinomycetes (*Streptomyces, Actinomadura, Amycolatopsis, Kribbella* and *Microbispora*) which had the ability to inhibit pathogenic bacteria. Besides that, endophytic actinomycetes have been shown to protect plants against different soil-borne plant pathogens including *Rhizoctonia solani* and *Verticillium dahliae* (Krechel *et al.,* 2002), *Plectosporium tabacinum* (El-Tarabily, 2003), *Gaeumannomyces graminis var. tritici* and *R. solani* (Coombs *et al.,* 2004) and *Fusarium oxysporum* (Cao *et al.,* 2005).

In terms of agricultural practice, attention has been paid to the possibility that actinomycetes can protect roots by inhibiting the development of potential fungal pathogens by producing enzymes which degrade the fungal cell walls and the production of antifungal compounds (Ilic *et al.,* 2007; Prapagdee *et al.,* 2008). Various *Streptomyces* species have been isolated, selected and developed for controlling

diseases and insects of plants since the early 1940s (Fravel, 2005). For example, *Streptomyces* sp. strain 5406 has been used in China to protect cotton crops against soil-borne pathogens (Valois *et al.*, 1996). Jinggangmycin, an antibiotic produced by *S. hygroscopicus* var. *jinggangensis*, is widely used for control of sheath blight of rice caused by *Rhizoctonia solani* in China (Shen, 1996). The commercial product, Mycostop, based on *S. griseoviridis* K61 and *S. lydicus* WYEC108, can control some root rot and wilt diseases caused by *Pythium* spp., *Fusarium* spp., *Rhizoctonia* spp. and *Phytophthora* spp. (Mahadevan and Crawford, 1997).

2.2 *Hylocereus* spp. and their diseases

Hylocereus spp. is a group of tropical epiphytic cacti commonly known as pitaya or pitahaya (Latin America) (Le Bellec *et al.*, 2006), strawberry pear and night-blooming cereus (English) (Mizrahi *et al.*, 1997), nanettikafruit or thanh long (Vietnam) (N'Guyen, 1996), and mata naga (Malaysia) (Cheah and Zulkarnain, 2008; Masyahit *et al.*, 2009b). This species originated from North, Central and South America (Britton and Rose, 1963; Barbeau, 1990). Since its introduction in Asia, by the French, 100 years ago, Vietnam has been reported as one of the biggest commercial producer of the fruit (McMahon, 2003). Generally, there are three varieties of dragon fruit which are classified into *Hylocereus polyrhizus* (red-fleshed with scarlet skin)(Table 2.2), *Hylocereus undatus* (white-fleshed with scarlet skin) and *Selenicereus megalanthus* (white-fleshed with yellow skin) (Hamidah and Zainudin, 2007; Halimi and Satar, 2007). In Malaysia, though, only *H. polyrhizus* and *H. undatus* are commercially viable and cultivated.

Hylocereus spp. is generally characterised as climbing plants, with aerial roots, that bear a glabrous berry with large scales (Fournet, 2002). This plant prefers a dry tropical or subtropical climate with an average temperature of 21-29 ºC, but can tolerate

temperatures of 38-40 ºC, and as low as 0 ºC for short periods. Rainfall requirements are 600-1300 mm with alternating wet and dry seasons. Though it likes a lot of sunshine, high levels of light intensity can cause damage to the plant (Luders and McMahon, 2006).

This plant attracts a lot of attention due to its nutritional properties and health benefits which have been well studied throughout the world. For example, the red pigments of *H. polyrhizus* were reported to contain betanin, betacyanin and lycopene (Wu *et al.,* 2006; Herbach *et al.,* 2006b). These compounds which are together known as anthocyanin (a type of antioxidant) are good for the body metabolism. Several other studies on the phytochemistry of *H. polyrhizus* have showed that consumption of this fruit boosts the immune system, aids in digestion and blood circulation, neutralise toxins in the body, as well as reduce the cholesterol level in the blood (Kow and Rokiah, 2005).

Table 2.2: Morphological features of *Hylocereus polyrhizus* (Halimi and Satar, 2007).

Parts	Description
Stem 	• green coloured stems • stem with triangular cross-section • more spines compared to other species
Flower 	• the margin of flowers with reddish perianth segments
Fruit 	• scarlet skin, red-fleshed, black seeds • red, wide, short, close arrangement of scales • oblong, 350-600g • 13.7% of brix (sweetness)

Hylocereus polyrhizus, like many other economically important plants, is affected by an array of diseases that lead to heavy losses and closure of several farms in Malaysia. The list of isolated pathogens based on literature reports has been presented in Table 2.3.

Table 2.3: List of *Hylocereus* spp. pathogens and their symptoms.

Pathogen	Symptom	Reference
Xanthomonas campestris	severe stem rot	Barbeau, 1990; N'Guyen, 1996; Crane and Balerdi, 2005; Le Bellec *et al.*, 2006; Hamidah and Zainudin, 2007; Halimi and Satar, 2007; Paull, 2007
Erwinia caratovora	water soaked lesion and subsequently becoming a soft rot	Barbeau, 1990; N'Guyen, 1996; Kostov and Ngan, 2006; Le Bellec *et al.*, 2006; Cheah and Zulkarnain, 2008
Colletotrichum gloeosporioides	anthracnose (brownish to yellowish lesions with chlorotic haloes and the formation of conidia in ascervuli)	Halimi and Satar, 2007; Masratul Hawa *et al.*, 2008b; Masyahit *et al.*, 2009b
Fusarium proliferatum	black to brownish lesions on stems of *H. polyrhizus*	Masratul Hawa *et al.*, 2008a
Fusarium oxysporum	basal rot of dragon fruit	Crane and Balerdi, 2005; Kostov and Ngan, 2006; Wright *et al.*, 2007
Dothiorella spp.	brown spots on stems and fruits of *Hylocereus* spp.	Zee *et al.*, 2004; Crane and Balerdi, 2005; Le Bellec *et al.*, 2006; Hamidah and Zainudin, 2007; Halimi and Satar, 2007
Cactus Virus X	chlorotic symptoms to *Hylocereus* stems	Boyle *et al.*, 1997; Liou *et al.*, 2001

2.3 Economically important pathogenic fungi – *Fusarium* spp.

The genus *Fusarium*, primary characterised by the presence of canoe- or banana-shaped conidia, was first introduced by Link in 1809 (Leslie and Summerell, 2006). This genus is a member of the Ascomycetes family and is known as fungi imperfecti due to their lack of sexual state (Fincham *et al.*, 1979). *Fusarium* spp. has some of the most economically important species plant pathogens that affect the agricultural industry worldwide. It also encompasses several other species which produce mycotoxins and are pathogenic to humans (Summerell *et al*., 2010).

The identification and classification of this complex and polyphyletic group have been problematic due to the variations in classification systems used by researchers worldwide. Species numbers ranging from over a 1000 were recorded in the early 1900s to as few as nine in the 1950s and 1960s and currently lies somewhere from 100 to 500 (Kirk *et al*., 2008; Leslie and Summerell, 2006). In 2006, Leslie and Summerell published a laboratory manual that describes 70 different species of *Fusarium*. This manual was the first *Fusarium* classification system where the description of new species was based on morphological characters, genetic and phylogenetic information. The morphological taxonomy of species in this genus is predominantly based on the form and abundance of their asexual reproductive structures (chlamydospores, phialides, macroconidia and microconidia) and on their cultural characteristics (colony texture, colour and aroma) (Booth, 1971; Nelson *et al*., 1983: Nelson *et al*., 1991; Gordon and Martyn, 1997; Edel *et al*., 2000; Llorens *et al*., 2006). *Fusarium* spp. produces three types of asexual spores which are microconidia, macroconidia and chlamydospores (Agrios, 1988). The most profuse and frequently produced spore under all conditions is microconidia, which are one or two-celled. Macroconidia, frequently seen on the surface of infected plants, are three-or more celled with pointed and curved ends. Micro- and macroconidia are formed for short-term

survival and dispersal. Chlamydospores, viable, thick-walled spores, filled with lipid-like material, are only formed for long-time survival in the soil when the host plant is not available (Agrios, 1997).

Fusarium species are found in tropical and temperate regions and are widely distributed in soil, subterranean and aerial plant parts, plant debris and other organic substrates (Nelson *et al*., 1994). Nevertheless, many *Fusarium* species are more commonly found in fertile cultivated and rangeland soils than in forest soils (Burgess *et al.,* 1975; Burgess *et al.,* 1988; Jeschke *et al.,* 1990). These fungi can be soil-borne, air-borne, or carried in plant residue, and can be recovered from any part of a plant (Leslie and Summerell, 2006). Leslie and Summerell (2006) reported that most plant species are susceptible to at least one *Fusarium*-associated disease. *Fusarium oxysporum* Schlechtendahl as emended by Synder and Hansen is one of the most economically important strain which consist of both pathogenic and non-pathogenic strains (Gordon and Martyn, 1997). The pathogenic variants are separated into special forms or *formae speciales* (plant species on which the disease is formed) and into races (crop cultivar specificity). Currently, more than 150 *formae speciales* have been described worldwide (Baayen *et al*., 2000; Hawksworth *et al*., 1995; O'Donnell *et al*., 2009; O'Donnell and Cigelnik, 1999). Zitter (1998) reported that mildly acidic pH (pH 5-5.5), high nitrogen and low levels of calcium and potassium in soil can induce disease. One of the biggest impacts of *Fusarium* ever recorded globally was on banana (*Musa* spp.) by Panama wilt disease caused by *Fusarium oxysporum* f. sp *cubense* which almost paralysed the commercial banana industry in the 1960 (Ploetz, 2000). To date, *Fusarium* wilt is still threatening banana production in many Cavendish-producing countries of the world (Ploetz, 2005a). In Malaysia, at least 43 *Fusarium* species have been isolated and identified from various economically important crops (Salleh, 2007). Table 2.4 shows the list of diseases, hosts and the disease inflicting *Fusarium* spp.. Apart from the

pathogenicity, *Fusarium* also contaminates harvested crops by producing mycotoxins, such as fusaric acid, diacetoxyscirpenol, T-2 toxin and zearalenone (Chakrabarti and Ghosal, 1987; Notz *et al*., 2002). Allergic symptoms and cancer symptoms may arise from the consumption these contaminated crops contaminated (Nelson *et al*., 1994).

Table 2.4: Diseases caused by *Fusarium* spp. in commercially important plants in Malaysia (Source: Salleh, 2007).

Disease (host)	***Fusarium* species**
Slanting death (tobacco)	*F. oxysporum, F.solani*
Bakanae (rice)	*F. fujikuroi*
Crown and root rot (asparagus)	*F. oxysporum, F. proliferatum*
Slow decline (pepper yellows)	*F. solani*
Vascular wilts (banana), Crown rot (banana)	*F. oxysporum* f.sp. *cubense*, *Fusarium* spp.
Vascular wilts (watermelon)	*F. oxysporum* f.sp. *niveum*
Vascular wilts (roselle)	*F. oxysporum*
Pokkah boeng (sugarcane)	*F. sacchari*
Stalk, ear and kernel rot (maize)	*Fusarium* spp.
Vascular wilts (long bean)	*F. oxysporum*
Fruitlet core rot (pineapple)	*Fusarium* spp.
Canker (coffee)	*F. xylariodes*
Die-back (orchids)	*F. proliferatum*

2.4 Biocontrol and the need for biocontrol agents

Cook and Baker (1983) defined biological control as the reduction in the inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man. The challenges for products in managing disease are increasing. Consumer demand for year-round production of fresh vegetables or fruits with reduced or no pesticide/fungicide residue continues to grow following concerns over the potential impact of disease management on the environment and on consumer

health (Punja and Uthede, 2003). Cook and Baker (1983) cited that most widely used control measure for suppressing soil-borne diseases is the use of environmentally hazardous fungicidal treatment of seeds, seedlings or soils. There are numerous reports on the negative impacts of chemical pesticides. Some of them include decrease in biodiversity of the soil-inhabiting microorganisms; hazardous effects of pesticide leftover on the aquatic systems (Johnston, 1986); the non-target environmental effects and the development of resistance to fungicides by pathogens (de Weger *et al.*, 1995; Gerhadson, 2002); severe health problems resulting from exposure of farmers to chemical pesticides (Arcury and Quandt, 2003); pesticide deposits in many food crops including fruits and vegetables which endanger the health of the consumers; furthermore, the increasing cost of pesticides, particularly in low-income countries of the world (Gerhadson, 2002). Naturally occurring microorganisms that are antagonistic to crop pathogens, have the potential to protect crop against the harmful effect of the pathogen as well as promoting the growth of plants, provide an alternative to chemical fungicides (Weller *et al.*, 2002; Welbaum *et al.*, 2004; Mark *et al.*, 2006). The usage of microbial antagonists as biological control agents is generally regarded safer than the chemical pesticides not only to the environment but also to the consumers of agricultural products.

2.5 Mechanisms of biocontrol agents

Many reports on disease management have pointed out on the different mechanisms involved in disease control. Therefore to successfully utilise biological control as disease management strategy, it is important to fully understand the mechanisms of disease reduction by these biocontrol agents. Some of the recognised mechanisms of biocontrol of pathogens will be discussed next.

2.5.1 Competition

Rhizosphere microorganisms commonly compete for resources such as nutrients, oxygen and colonisation site in soil. In biological control, competition occurs when antagonist directly compete with pathogens for these resources. According to Alabouvette *et al.* (2006), competition for nutrients, especially for carbon, is assumed to be responsible for the phenomenon of fungistasis characterising the inhibition of fungal spore germination in soil. Apart from that, competition for trace elements, such as iron, copper, zinc, manganese etc., also happens in soils. For instance, iron is an essential growth element for living organisms and the lack of its bio-available form in soil habitats results in a concerted competition (Loper and Henkels**,** 1997). Siderophores, low molecular weight compounds with high iron affinity, are produced by some microorganisms (also by most biocontrol agents). It solubilises and competitively obtains ferric ion under iron-limiting conditions, thereby making iron unavailable to other soil microorganisms which cannot grow without it and at the same time favours rapid growth of the producing organisms (Loper and Henkels**,** 1997; Haas and Défago, 2005). Pyoverdin, salicylic acid and pyochelin are examples of siderophores produced by biocontrol agents (Haas and Défago, 2005). Another function of siderophores is as good chelators of some elements other than iron. Subsequently, when these elements are increasingly made available to the bacteria, siderophores may directly stimulate the production of other anti-microbial compounds (Duffy and Défago, 1999). Siderophores can also function as a diffusible bacteriostatic or fungistatic antibiotic under certain conditions (Haas and Défago, 2005).

2.5.2 Parasitism

The process initiated by physical destruction of the fungal cell wall mediated by the action of hydrolytic enzymes produced by a biocontrol agent is defined as

mycoparasitism (Adams, 1990). The two major structural components of most plant pathogenic fungi are chitin and ß-1,3-glucan. Excretion of extracellular enzymes enables the antagonists to invade pathogens causing lyse of pathogen cell walls or degradation of chlamydospores, oospores, conidia, sporangia, and zoospores. These extracellular enzymes include chitinases, cellulases, proteases and ß-1,3-glucanases. Dunne *et al.* (2000) demonstrated that overproduction of extracellular protease in the mutant strains of *Stenotrophomonas maltophilia* W81 resulted in improved biocontrol of *Pythium ultimum.*

2.5.3 Induction of plant resistance mechanisms

All plants express natural defence reactions against stresses from biotic or abiotic factors such physical stresses (extreme temperatures), inoculation by pathogenic or non-pathogenic organisms, or even chemical molecules from natural or synthetic origins (Alabouvette *et al*., 2006). One of the mechanisms involved in elicitation of plant defence reactions is the early recognition of the aggressor by the plant (Lugtenberg *et al.*, 2002). A cascade of molecular signals and the transcription of many genes are instantly initiated by the recognition. This eventually results in the production of defence molecules such as phytoalexins, pathogenesis-related (PR) proteins (such as chitinases, ß-1, 3-glucanases, proteinase inhibitors) and reinforcement of cell walls by the plant (Van Loon, 2000; Whipps, 2001). Cell wall thickenings, wall appositions or rapid death of the injured plant cells resulting in necrosis of the immediate adjacent tissues are barriers which cut the pathogen off its nutrients and contribute to slowing down of the fungus progressive invasion (Lugtenberg *et al.,* 2002; Alabouvette *et al*, 2006). A virulent pathogen naturally inhibits resistance reactions, or sidesteps the effects of active defences. As a result of these natural defence mechanisms, plants are able to produce an immune response after a primary pathogen infection known as

systemic acquired resistance (SAR). The host plant can also benefit directly from non-pathogenic rhizobacteria and fungi through the production of metabolites that either stimulate root development and plant growth or trigger the induction of systemic resistance (ISR) that is phenotypically similar to SAR (Van Loon *et al.,* 1998; Bakker *et al.,* 2003). In other words, SAR is a pathogen-induced type of resistance which requires accumulation of salicylic acid while ISR is a rhizobacteria-induced type that depends on responses to ethylene and jasmonic acid (Bakker *et al.,* 2003). These plant defence-inducing bacteria are also known to enhance plant growth and are referred to as plant growth promoting rhizobacteria (PGPR).

2.6 Plant growth promoting rhizobacteria (PGPR)

Free living, soil-borne bacteria isolated from the rhizosphere are generally known as plant growth-promoting rhizobacteria (PGPR). The exploitation of microorganisms with the intention of enhancing nutrients accessibility for plants is an important practice and a necessity in agriculture field (Freitas *et al*., 2007). Kloepper (1993) reported that certain plant growth-promoting rhizobacteria (PGPR) are able to function as biological control agents and some biocontrol agents can suppress plant pathogens and successively stimulate plant growth. Recent investigations on PGPR revealed that it can promote plant growth mainly by following means; (1) producing ACC deaminase to reduce the level of ethylene in the roots of developing plants (Dey *et al.,* 2004), (2) producing plant growth regulators like indole acetic acid (IAA) (Mishra *et al*., 2010), gibberellic acid (Narula *et al*., 2006), cytokinins (Castro *et al*., 2008) and ethylene (Saleem *et al*., 2007), (3) asymbiotic nitrogen fixation (Ardakani *et al*., 2010), (4) exhibition of antagonistic activity against phytopathogenic microorganisms by producing siderophores, β-1,3-glucanase, chitinases, antibiotics, fluorescent pigment and cyanide (Pathma *et al*., 2011) and (5) solubilisation of mineral phosphates and other

nutrients (Hayat *et al*., 2010). Nevertheless, the mechanisms involved in biological control, either to control pathogens or to stimulate plant growth, are highly dependent on strain, host plant, pathogens as well as environment factors.

One of the first developments in the field of PGPR took place in the 1970s whereby several experiments established the capability of *Pseudomonas* strains in controlling soil-borne pathogens and indirectly enhancing plant growth and increasing the yield of potato and radish plants (Howie and Echandi, 1983). Recently, the potential of non-streptomycete actinomycetes to solubilise insoluble phosphates in soil and to promote plant growth has been investigated (El-Tarabily *et al.*, 2008). An isolate of *Micromonospora endolithica* was found able to solubilise considerable amounts of phosphate (P), to produce acid and alkaline phosphatases as well as several organic acids and promote the growth of beans despite the inability to produce any stimulatory compounds (such as auxin, cytokinin, and gibberellin). Apart from that, other actinomycetes strains such as *Micromonospora* spp, *Streptomyces* spp., *Streptosporangium* spp., and *Thermobifida* spp. were able to colonise the plant rhizosphere extensively, thus presenting a huge potential as biocontrol agent against a range of root pathogenic fungi (Franco-Correa *et al*., 2010). de Vasconcellos and Cardoso (2009) reported a rhizospheric *Streptomyces* as potential biocontrol agent of *Fusarium* and *Armillaria* pine rot and as PGPR of *Pinus taeda*.

2.7 Actinomycetes related researches in Malaysia

In Malaysia, the research interest in this microorganism is slowly increasing. There have been a number of reports on the isolation and biocontrol assays using actinomycetes. Actinomycetes have been isolated from a wide variety of sources such as soil (Al-Tai *et al.*, 1999), marine organisms (Tan, 2006; Mahyudin, 2008), plants (Becker, 1983; Zin *et al.*, 2007; Ghadin *et al.*, 2008), agriculture soils (Jeffrey, 2008), tropical rainforests soil

(Numata and Nimura, 2003), and primary dipterocarp forest soil (Ho *et al.,* 2001; Nakajima *et al.,* 2003). In the aspect of biocontrol assays, Getha (2005) reported isolation of *Streptomyces* sp. strain g10 which was active against a number of pathogenic fungi such as *Fusarium oxysporum, Phytophtora palmivora, Pyricularia oryzae* and *Rhizoctonia solani,* and produced a number of secondary metabolites such as geldanamycin, desferrioxamine E (nocardamine) and elaiophylin. Besides that, *Micromonospora* sp. M39, an isolate from mangrove rhizosphere soil in West Malaysia, produced metabolites that are effective against the rice blast pathogen *Pyricularia oryza* MPO 292 (Ismet *et al.,* 2004). Compounds such as 2, 3-dihydroxybenzoic acid, phenylacetic acid, and the antibiotics, cervinomycin A1 and A2 were found in the crude extract. A number of novel antibiotics were also reported. Langkolide, a macrolactone antibiotic was isolated from *Streptomyces* sp. Acta 3062, which was isolated from the rhizosphere soil collected from the roots of *Clitorea* sp. at Burau Bay in the tropical rain forest of Langkawi, an island at the north-west coast of Malaysia (Helaly *et al.,* 2012). A new family of metabolites named gombapyrones, were elucidated from *Streptomyces griseoruber* Acta 3662 which was isolated from soil collected from the rhizosphere of bamboo trees in the tropical rainforest of the University of Malaya Field Station at Gombak, Selangor, Malaysia (Helaly *et al.,* 2009). These publications show the possibility that Malaysian ecosystem inhabits a wide range of actinomycetes population which remains untapped.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Sampling site

The sampling sites were two *Hylocereus polyrhizus* plantation areas in the township of Kuala Pilah in Negeri Sembilan. These sites were affected by outbreak of diseases which began to badly influence the production of dragon fruits. Department of Agriculture of Negeri Sembilan identified this outbreak as a worrying sign, therefore requested assistance from our university to overcome it.

3.2 Collection of soil samples

The soil samples for this study were collected from the rhizosphere of the plants about ten to fifteen centimeters below the soil surface. First, the soil around the root area was cleared using a spade. Soil samples within close proximity of the roots were collected using a sterile cork borer. In addition, soil particles attached to the excised roots were also collected. Soils samples were taken randomly from 10 different areas of each sampling site. All soil samples were stored in sterile universal containers and labelled accordingly.

3.3 Collection of diseased *Hylocereus polyrhizus* stems

Thirty eight diseased *Hylocereus polyrhizus* stems tissues were collected from 15 plants in each sampling site. To reduce cross-contamination, the plant cutter was wiped with ethanol (70%, v/v) before excising each stem. Excised stems were kept in plastic bags, sealed and labelled accordingly.

3.4 Isolation of actinomycetes from soil samples

The soil samples collected from the sampling site were air-dried in sterile glass Petri dishes at room temperature for three days prior to isolation using moist-heat pre-treatment. For this pre-treatment, one gram of air-dried soil sample was mixed with nine ml of sterile yeast-extract-sodium dodecyl sulphate (SDS) solution (Hayakawa and Nonomura, 1989; Appendix A1). The soil suspension was sonicated for 30 minutes and then incubated in water bath at 45 ± 2°C for 30 minutes. After incubation, the suspension was serially diluted to a final concentration of 10^{-3} and 0.1 ml of the dilution were aseptically spread-plated on starch-casein agar (SCA; Küster and Williams, 1964; Appendix A2), humic acid-vitamin agar (HVA; Hayakawa and Nonomura, 1987; Appendix A3) and raffinose-histidine agar (RHA; Vickers *et al*., 1984; Appendix A4). All isolation media were supplemented with cycloheximide (50µg/ml), nystatin (50µg/ml) and nalidixic acid (20µg/ml) to reduce the growth of fungal and bacterial contaminants. Sample dilution plates were incubated at 28 ± 2°C for two to four weeks until sporulated or non-sporulated actinomycetes colonies were observed. Isolates which were putative actinomycetes were inoculated onto yeast extract-malt extract agar (ISP 2; Appendix A5), inorganic salts-starch agar (ISP 4; Appendix A6) and sporulation agar (SA; Appendix A7) for purification. Axenic cultures were kept as spore suspension and mycelia fragments on small plug in 20% (v/v) glycerol at -20° C for preservation and on ISP 2 agar plates at 4° C for routine use.

3.5 Isolation of pathogenic agents from *Hylocereus polyrhizus* stems

Lesions from the stem region of *Hylocereus polyrhizus* were excised aseptically into approximately 5 mm^2 pieces using scalpel. The excised pieces were rinsed in 0.1% (v/v) Tween 20 for thirty seconds, followed by 1% (v/v) sodium hypochlorite for five minutes and finally washed in sterile distilled water for five minutes. The tissue pieces were then

surface sterilised in 70% (v/v) ethanol for five minutes and air-dried in a laminar flow chamber (Taechowisan *et al*., 2003). The surface-sterilised lesion tissues were then placed on potato dextrose agar (PDA; Appendix A8) plates and incubated at 25 ± 2 °C. Growing edges of the mycelia emerging from the tissue were sub-cultured onto fresh PDA plates to obtain axenic cultures of the pathogens.

The axenic cultures were maintained on agar slants at 25 ± 2 °C for routine use and on 2% (v/v) water agar (WA; Appendix A9) at 4 ± 2 °C as stock culture. Slides of the axenic cultures were also prepared to observe the cultural characteristics using a light microscope. Species level identification was done with the help of Dr. Baharuddin Salleh from Universiti Sains Malaysia (USM).

3.6 Primary screening of actinomycetes isolates (Qualitative assay)

The 162 actinomycetes isolated from the soil samples were screened for their potential suppressive activity against fungal pathogens isolated from the stems of *Hylocereus polyrhizus* using the cross-plug assay (Getha and Vikineswary, 2002). As both actinomycetes and fungal strains grew well on ISP 2 media, this media was selected for this bioassay.

A five millimeter (mm) diameter agar plug of ten days-old actinomycetes isolate from ISP 2 agar was positioned at the centre of the assay plate. Two five-mm diameter agar plugs of seven days-old fungal isolate were transferred at equidistant positions to the actinomycete plug and incubated at 28 ± 2°C for five days. Three replicates were carried out for each of the presumptive antagonist strains and the phytopathogenic fungi. The presence of inhibition zone and inhibition widths were observed and compared to the control plates. Test plates with fungal pathogen plugs only were prepared as control.

3.7 Quantitative screening of selected actinomycete isolates

Based on the primary screening, twenty three isolates showed inhibitory activity against at least one test pathogen. These isolates were further screened using agar streak assay (Crawford *et al.*, 1993) to quantitatively measure their ability to inhibit the test pathogens. Actinomycete was streak-inoculated two centimeters from the rim of ISP 2 plate and incubated at 28 ± 2°C for five days. After five days, a plug (5mm diameter) from the edge of actively growing seven days-old fungal pathogen colony was transferred to the centre of the test plate. Test plates with fungal pathogen plugs only were prepared as control. All test plates were prepared in triplicates, incubated at 28 ± 2°C for five days and subsequently examined for inhibition. The inhibition zone was recorded by measuring the fungal colony radius on the control plate (γ_0) and the distance of fungal colony growth perpendicular to the strain colonies on the test plate (γ). Percentage of fungal linear growth inhibition was calculated according formulation by Getha (2005).

$$\text{Percentage of inhibition of fungal linear growth} = [1 - (\gamma / \gamma_{0})] \times 100$$

3.8 Observation of inhibition zones through scanning electron microscopy (SEM)

Three isolates with the highest percentage of inhibition against all fungal pathogens, based on the agar streak assay, were studied for their resulting mechanisms of inhibition by scanning electron microscopy (SEM). Mycelial plugs were excised at the border where inhibition was observed. Excisions were made at different points of the inhibition zones on the assay plate to observe the variations in the mechanisms of inhibition. The excised plugs were prepared according to standard laboratory protocols (Appendix B1) for visualization through scanning electron microscopy (Getha, 2005).

3.9 Characterisation of selected actinomycetes strains

3.9.1 Micromorphology

Three actinomycete isolates with the best inhibitory activities in the quantitative agar streak assay were selected to perform their identification using selected genotypic and phenotypic characterisation methods. For the micromorphological characterisation, the spore structures and ornamentations of the selected strains were observed using SEM. The selected isolates were inoculated on duplicates of ISP 2 agar medium and were incubated at 28 ± 2°C for 10 days. Single colonies of the actinomycete isolate were excised using cork borer. The excised plugs were prepared according to standard laboratory protocols (Appendix B1) for visualisation through SEM. The spore structures and ornamentations observed were identified according to simple classification of sporophore-spore chain types proposed by Shirling and Gottlieb (1966).

3.9.2 Phenotypic characterisation of the selected strains

3.9.2.1 Cultural studies

Growth and cultural characteristics of the selected strains were observed on yeast-extract-malt extract agar (ISP 2), oatmeal agar (ISP 3; Appendix A10), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5; Appendix A11), peptone-yeast extract-iron (ISP 6; Appendix A12) and tyrosine agar (ISP 7; Appendix A13) after 10 days of incubation at 28 ± 2°C (Shirling and Gottlieb, 1966). Colors of aerial, substrate mycelia and diffusible pigments were determined using Methuen Handbook of Colour (Kornerup and Wanscher, 1963) and recorded. Production of melanoid pigment was observed by distinct discoloration (greenish–brown to brown or black diffusible pigment or distinct brown pigment) on ISP 6 and ISP 7 after four days of incubation at 28 ± 2°C (Shirling and Gottlieb, 1966).

3.9.3 Physiological characterisation of representative actinomycetes strains

3.9.3.1 Growth at different pH, temperature and salinity level

The pH range for growth of the selected isolates was tested by inoculating them onto triplicates of ISP 2 agar medium with adjusted pH. The pH was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 with 1 M HCl or 1 M NaOH. The test plates were incubated for two weeks at 28 ± 2°C (Williams *et al*., 1983).

The temperature range for the growth of the selected isolates was determined by inoculating them onto triplicates of ISP 2 agar medium and incubating them at five different temperature settings (4°C, 10°C, 18°C, 38°C and 45°C). Actinomycete growth at 45°C were observed after 14 days while growth at 4°C and 10°C were observed after six weeks of incubation (Williams *et al*., 1983). For the rest of the temperatures, observation was done after 10 days.

The tolerance of selected isolates to increasing salinity level was determined by inoculating them onto triplicates of ISP 2 agar medium with adjusted sodium chloride concentration. The sodium chloride concentrations were adjusted to weight to volume (w/v) ratio of 2%, 4%, 6%, 8% and 10% (Okazaki and Okami, 1975). The inoculated plates were incubated for ten days at 28 ± 2°C and subsequently the growth of isolates in comparison to the control plates (without sodium chloride) was recorded.

3.9.3.2 Utilisation of sole carbon sources

Carbon utilisation test was conducted by inoculating selected isolates on basal medium of Pridham-Gottlieb (ISP 9; Appendix A15) agar which were added with filter-sterilised carbon sources adjusted to the final concentration of 1% (w/v) (Shirling and Gottlieb, 1966). The carbon sources used in this experiment were arabinose, mannitol, maltose, raffinose, sorbitol, galactose, mannose, sucrose, rhamnose, xylose, lactose, ribose,

inositol and glucose. ISP 9 agar with glucose served as the positive control and ISP 9 agar without any carbon sources served as negative control in this experiment. The inoculated plates were incubated at 28 ± 2°C for 10 to 16 days before the results were scored. This test was conducted in triplicates.

3.9.3.4 Susceptibility to antibiotics

Susceptibility of the selected isolated to different antibiotics (kanamycin, gentamicin, neomycin, erythromycin, streptomycin, chloramphenicol, novobiocin, tetracycline and ampicillin) at defined concentrations was determined using the method of Rheims *et al*. (1998). Sterile cotton buds were used to swab the aerial spores of a ten days old culture to evenly lawn onto ISP 2 agar medium. Antibiotic sensitivity discs were placed centrally on the lawned plates and incubated at 28 ± 2°C for four days. The subsequent inhibition zones (if any) were scored.

3.9.3.5 Degradation activity

The degradation of casein (1%, w/v, skim milk) was identified using Nutrient agar (NA; Appendix A16), as the basal medium. A five-mm plug was taken from a ten days old culture and placed centrally on the test plate. The plates were incubated at 28 ± 2°C. Clearing zone (if any) around the growth area was observed after seven days of incubation (Williams *et al*., 1983).

The degradation of L-tyrosine (0.5%, w/v), xylan (0.4%, w/v) and xanthine (1.0%, w/v,) were determined using NA, as the basal medium. A five mm plug was taken from a ten days old culture and placed centrally on each test plate. The plates were incubated at 28 ± 2 °C and checked for clearing zones (if any) after 7, 14 and 21 days (William *et al*., 1983).

Liquefaction of gelatin was tested in 12% (w/v) gelatin media prepared in McCartney bottles. Three five-mm plugs were taken from a ten days olds culture and dropped into the bottles. After a week of incubation at 28 ± 2 °C, the bottles were placed in refrigerator at 4 ± 2°C for one hour. The bottles were then inverted. Liquefied gelatin indicated a positive reading when compared to the gelatin in control bottles with no actinomycetes plugs which was solid.

Nitrate reduction test was conducted using a kit (BioMerieux, France) according to manufacturer's protocol.

3.9.4 Molecular identification of potential strains

3.9.4.1 DNA extraction of selected actinomycetes

The selected isolates were cultured in non-sporulation agar (NSA; Sanglier *et al*., 1993; Appendix A14) plates and incubated at 28 ± 2°C for two to three days before the formation of dense spores. A small cell pellet of biomass was scraped from culture for the DNA extraction. The total genomic DNA for the selected isolates was extracted using a genomic DNA extraction kit (NucleoSpin®, Appendix B2). The eluted DNA was stored at -20°C. To ensure that DNA extraction was successful, the extracted DNA was analyzed by agarose gel electrophoresis (Appendix B3).

3.9.4.2 PCR amplification of extracted actinomycetes DNA

The DNA extractions of the selected isolates were used as templates in PCR reaction. Reactions were performed in a final volume of 50µl consisting of 0.2 mM of each of the four dNTPs (deoxynucleotide triphosphates, Promega), 0.2µM of forward and reverse primers (27f and 1525r, 1st BASE), 1µl extracted DNA, 1.5 mM $MgCl_2$ (Promega), Taq

polymerase (Promega), 5µl of 10X reaction buffer and 39.25µl of sterile distilled water. Control reaction without bacterial DNA was included with each PCR experiment. Amplification was performed using ESCO's Swift™ Maxi Thermal Cycler according to the following profile: initial heating of 1 min at 95 °C, followed by 30 cycles of denaturation (1 min at 95 °C), primary annealing (1 min at 52°C), extension (1 min at 72 °C) and a final extension step at 72 °C for 10 min. The amplified fragments were either stored at -20°C or analyzed by agarose gel electrophoresis. Successfully amplified PCR products were purified using the QIAquick Purification Kit (Qiagen) following manufacturer's protocol (Appendix B4). The purified PCR products were analyzed by agarose gel electrophoresis.

Bidirectional sequencing of the PCR product using the forward and reverse primers was done by First Base, Malaysia. Using the sequences obtained, homology search was performed through the standard BLAST sequence similarity searching program from the web server (http://www.ncbi.nlm.nih.gov/BLAST/) against previously reported sequence at the GenBank/EMBL/DDBJ database. The sequence was multiply aligned with selected sequences obtained from Genbank/EMBL/DDBJ by using the CLUSTALW (Thompson *et al*., 1994). The alignment was manually verified and adjusted prior to the construction of phylogenetic tree. The phylogenetic tree was constructed by using maximum composite likelihood method (Tamura *et al*., 2004) in the MEGA4 (Tamura *et al*., 2007). The confidence values of branches of the phylogenetic tree were determined using the bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The values for sequences similarity among the closest strains were calculated manually.

3.10 Characterisation of actinomycete crude extracts by HPLC-DAD-UV analysis

3.10.1 Preparation of actinomycete crude extracts

Selected actinomycetes isolates were streaked fully on ISP 2 media and incubated at 28 ± 2 °C for seven days. One hundred fifty plates were prepared for each isolate used. After seven days, the agar with the cultures were cut into small pieces using scalpel and transferred in 500ml of Erlenmeyer's flask and filled with ethyl acetate covering the cultures. The solvent-culture mixture was left to soak in ethyl acetate for 48 to 72 hours in rotary shaker at 100rpm for complete extraction. The mixture was then filtered through Whatman No.4 filter paper and the filtrate was evaporated in a rotary evaporator (BUCHI) at 40°C. The crude extracts was completely dried in a water bath at 50°C and transferred into autosampler vials. The final weight of the crude extracts were weighed and stored at -20°C.

3.10.2 Analysis of actinomycetes crude extracts using HPLC-UV-DAD technology

The crude extracts were analysed using a reversed-phase high-performance liquid chromatography (HPLC), under standardised chromatograph conditions, coupled with computerised diode-array detection (Fiedler, 1993) at the Mikrobiologisches Institut of Universität Tuebingen, Germany. Each crude extracts were dissolved in methanol to a concentration of 25mg/ml and 10 µl of the sample was then injected into HPLC column (Novogrom stainless steel column; 125 X 4.6mm) which was fitted with a guard column (20 X 4.6mm) and filled with 5 µm Nucleosil-100 C-18 (Grom, Herrenberg, Germany). The chromatographic system consisted of HP 1090M liquid chromatograph with built-in diode array detector, HP 79994A Pascal-work station (200MB hard disk) and HP 79988A software rev. 5.3 (Hewlett Packard, Waldbronn, Germany). The samples were separated by linear gradient elution using 0.1% *ortho*-phosphoric acid (Merck,

analytical-reagent grade) and acetonitrile (Merck, HPLC grade) as the mobile phase at a flow-rate of 2ml/min. The gradient was from 0% to 100% acetonitrile in 15 min after a 5 min post-time at 100% ortho-phosphoric acid, followed by a 1-min hold at 100% acetonitrile. Multiple wavelength monitoring was performed at 210, 230, 240, 260, 310, 360 and 435 nm, and 500nm, without reference wavelength. The spectrum range was obtained from the range 200 to 600 nm with a sampling interval of 640 milliseconds. The UV-visible absorbance spectra of the crude extracts of the selected isolates were compared with the retention times and UV-visible spectral data from known antibiotics and other metabolites in natural products libraries (Fiedler, 1993). Comparison of the data points of two UV spectra resulted in a match factor. A factor of zero indicated no match and 1000 indicated identical spectra. Similar spectra showed a value above 990 and values below 900 indicated different compounds (Huber, 1989; Fiedler and Kohn, 1993).

3.11 Phytotoxicity test

3.11.1 Surface sterilisation of maize seeds

The maize seeds used for this experiment were obtained from a local hypermarket. The seeds were soaked overnight in distilled water. The distilled water was then drained out and the seeds were then soaked in 2% sodium hypochlorite for five minutes. The seeds were then rinsed five times with sterile distilled water prior to use (Bressan, 2003).

3.11.2 Seed viability test

Seed viability test were first conducted to test the quality of the seeds. This was done using the paper towel method (Gholami *et al*., 2009). One hundred and twenty surface-sterilised seeds were placed on a damp paper towel in a plastic container and grown in

growth chamber at 28 ± 2 °C. After seven days, the number of seeds that germinated was counted.

3.11.3 Inoculum preparation

Ten ml of sterile distilled water was pipetted onto a 10 days old actinomycetes culture grown on ISP 2 agar. Using a sterile hockey stick, the aerial spore mass was scraped to form spore suspension. The spore suspension was then collected in a sterile centrifuge tube and centrifuged at 5000rpm for 10 minutes. Supernatants were removed and one ml of sterile distilled water was added and vortexed to resuspend the pellet. Serial dilution was done to determine the colony forming unit/ml (CFU/ml) of each isolate. Based on the CFU/ml of each isolate, the high dosage (1×10^8) and low dosage (1×10^6) were prepared by serial dilution method.

3.11.4 Phytotoxicity assay of selected antagonistic actinomycetes

For the phytotoxicity analysis, 30 ml of water agar was prepared and dispensed into plant tissue culture jars and autoclaved at 121°C for 15 minutes. Five ml of spore suspension was added to the cooled but yet to solidify agar medium. The substances were mixed gently in a swirling motion and left to solidify. Five surface-sterilised maize seeds were placed onto the agar medium in each jar. The seeds were left to germinate under 24 hours of fluorescent lighting for 10 days. Parameters such as plant height, main root length, number of leaves and number of secondary roots were recorded. The experiment was done in triplicates. Agar medium without addition of spore suspension served as the positive control in this experiment. Data were analyzed using SPSS 17.0 for Windows. Mean separation was accomplished using Duncan's Multiple Range Test. The statistical significance was determined at $P \leq 0.05$.

3.12 Greenhouse trial

For this experiment, 40 dragon fruit stems of 50 cm were used for each treatment. The end of each dragon fruit stem was excised at a slanted angle to promote rooting. The soil used to plant these dragon fruit stems was top soil which was sterilised at 121°C for 30 minutes. 20cm black polybags was used for planting purposes. Two types of treatments were chosen for this experiment, i.e., the first treatment was the control treatment where the excised end of the stem was dipped in sterile distilled water and planted in polybag with about one quarter of it embedded in sterilised topsoil mixed with 20ml of sterile distilled water beforehand. The second treatment was done by dipping the excised end of the stem with spore suspension of isolate K98 with the concentration adjusted to high dosage (1×10^8 CFU/ml). The treated stem was planted in polybag with about one quarter of it embedded in sterilised topsoil mixed with 20ml of high dosage spore suspension of isolate K98 beforehand. The planted stems were placed in a greenhouse and watered twice a week. Destructive sampling was done after 45 days and 90 days of growth. Parameters such as formation of lateral shoots, total length of lateral shoots, length of longest adventitious root, total number of adventitious roots and formation of new disease spots. Data were analyzed using SPSS 17.0 for Windows. Mean separation was accomplished using Duncan's Multiple Range Test. The statistical significance was determined at $P \leq 0.05$.

CHAPTER 4

4.0 RESULTS

4.1 Isolation of actinomycetes from soil samples

In this study, a total of 162 putative actinomycetes was isolated. These isolates can be tentatively divided into two groups which were streptomycete-like and non-streptomycete-like (Figure 4.1). Generally, isolates which were streptomycete-like grew with well-developed sporulating aerial hyphae on ISP 2, ISP 4 and sporulation agar; while isolates from the non-streptomycete-like showed optimal growth on ISP 2 and sporulation agar. One hundred and ten isolates (67.9%) were tentatively classified as streptomycete-like and 52 isolates (32.1%) were classified as non-streptomycete-like based on their ability to produce aerial mycelium on ISP 2 and ISP 4. Contamination on the isolation plates were minimal suggesting that the antibiotics added was at the correct combination and dosage to inhibit contaminants.

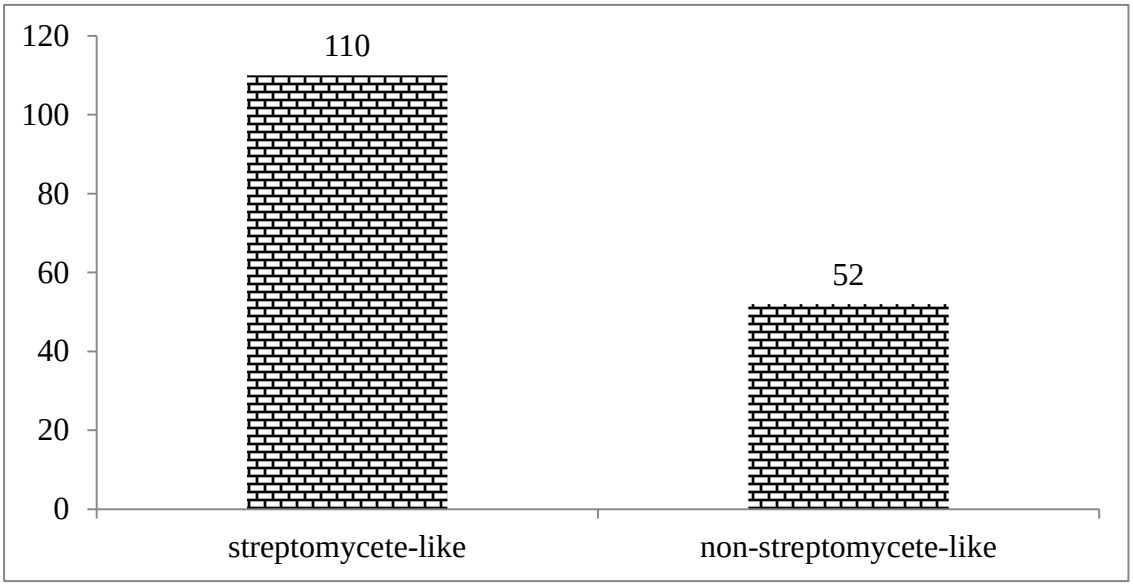

Figure 4.1: Number of isolates categorised into streptomycete-like and non-streptomycete-like groups according to visual observations of their growth on ISP 2, ISP 4 and SA media.

The putative actinomycetes were sorted into different series based on their aerial mycelium colour after culturing them on sporulation agar (SA) for 14 days. There were seven key aerial mycelium colour series which were grey, white, brown, orange, yellow, green and black. Different shades of the major colour series as well as substrate mycelium and soluble pigment colours were also recorded. Based on Table 4.1, majority of the isolates fell into the grey colour series (41.1%) followed by the white colour series (37.7 %). Isolates from all color series formed soluble pigments except from the orange and black colour series. Isolates from the orange or black colour groups were most probably from the *Micromonospora* spp. with their characteristic orange colour colonies turning to black mucoid mass during sporulation.

4.2 Isolation of pathogenic agents from diseased *Hylocereus polyrhizus* stems

Three pathogenic fungi were isolated from diseased regions of the *Hylocereus polyrhizus* stems in this study. Based on initial identification through light microscopy, these isolates were tentatively identified as *Fusarium* spp. and named as *Fusarium* 1 (Plate 4.1.1c), *Fusarium* 2 (Plate 4.1.2c) and *Fusarium* 3 (Plate 4.1.3c). All the isolates grew well when cultured in Potato Dextrose Agar (PDA, Difco). *Fusarium* 1 (Plate 4.1.1a) initially produced whitish aerial mycelium which turned purplish after 10 days. Meanwhile, *Fusarium* 2 (Plate 4.1.2a) had whitish to reddish aerial mycelium and *Fusarium* 3 (Plate 4.1.3a) had golden brown aerial mycelium. *Fusarium* 2 was a slow growing fungus compared to the other isolates and produced yellowish exudates after five days of incubation at 28 ± 2°C. Species level identification was done with the help of Dr. Baharuddin Salleh at the Fusarium Culture Collection Unit, Universiti Sains Malaysia (USM). The three isolates were finally identified as *Fusarium oxysporum* (*Fusarium* 1), *Fusarium decemcellulare* (*Fusarium* 2) and *Fusarium semitactum* (*Fusarium* 3).

Table 4.1: Isolates designated into major colour series according to the colour of their aerial mycelium based on their growth on ISP3 media. Shades of the aerial mycelium and colour of their substrate mycelium and soluble pigment were also recorded with reference to Methuen Handbook of Colour (Kornerup & Wanscher, 1963).

Colour series	Aerial mycelium shades	Substrate mycelium	Soluble pigment	Number of isolates
grey	light/medium/ dark grey, blue, green, brown, yellow, white, purple	pale/red/green/dark/ white/ golden/maroon brown, green, purple, pale yellow, white,	red/green/light/ dark/pale/purple brown, dark purple, green grey	67 (41.4%)
white	yellow, orange, brown, green	dark/light brown, white, pale yellow, orange, grey	light/pale/dark brown, pale yellow	61 (37.7%)
brown	light brown/brown, light grey/grey, white	grey, white, light brown, pale yellow	light/dark brown	13 (8.0%)
orange	black, pink, dark orange	light/dark orange, white, black	-	7 (4.3%)
yellow	pale yellow, green	pale yellow, brown, white, green	pale/dark brown, green	7 (4.3%)
green	yellow, grey	white, dark brown, grey	brown/dark brown	4 (2.5%)
black	black	black	-	3 (1.9%)

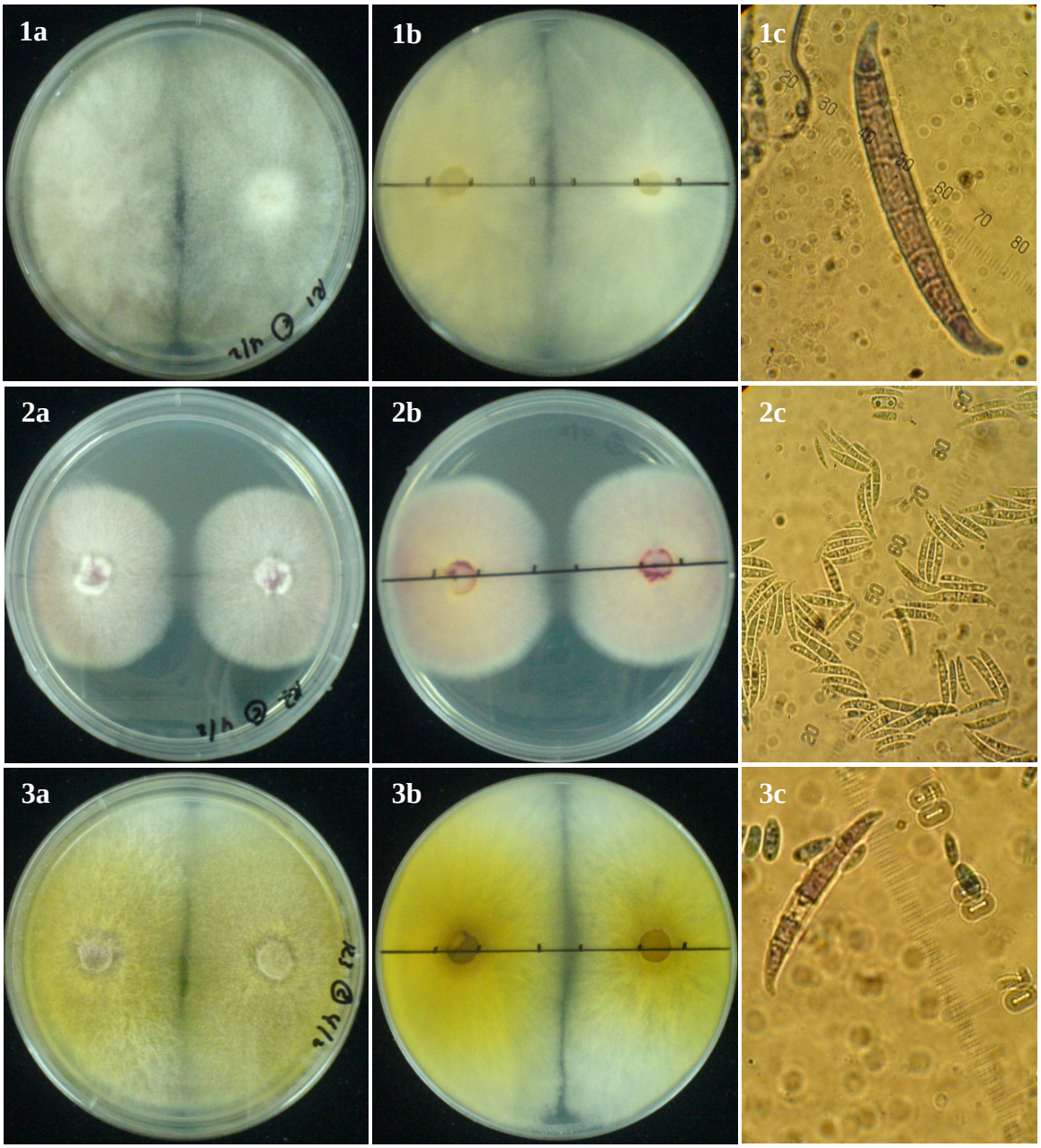

Plate 4.1: Cultural characteristics (a: aerial view, b: reverse side view) of the pathogenic fungi isolated after seven days of growth on PDA. Based on light microscopy (c), the fungi were tentatively identified as *Fusarium* spp. (1: *Fusarium oxysporum*; 2: *Fusarium decemcellulare*; 3: *Fusarium semitactum*)

4.3 Screening actinomycetes for antagonistic property

The cross-plug assay was conducted to eliminate actinomycetes which were not able to inhibit the fungal pathogens. This method was a faster way of screening a large number of actinomycetes isolates as the actinomycetes plug from a ten days old culture and the fungal plugs from a one week old culture were introduced together and incubated for 5 days to observe the inhibition. Inhibition zones were visible three days into the incubation period. These inhibition zones were categorised according to their respective strengths recorded by visual observation (qualitative). Very strong inhibition was indicated when the fungal mycelium growth was mainly around or on the fungal plug (Plate 4.2A). Strong inhibition was indicated when fungal mycelial growth was totally inhibited in the area facing the actinomycetes but moderate growth was observed on the other side of the fungal plug (Plate 4.2B). Moderate inhibition was indicated when the fungal mycelium growth facing the actinomycetes plug was limited but dense on the other side of the fungal plug (Plate 4.2C). In weak inhibition, the hyphal growth was only slightly stunted (Plate 4.2D). Plates with no inhibition were comparable to control plates (Plate 4.2E).

Out of the 162 actinomycetes isolated, 23 isolates suppressed at least one of the fungal isolate (Table 4.2). Majority of the antagonistic isolates (56.5%) were from the grey colour series (Figure 4.2). These isolates were streak-inoculated two centimetres from the rim of the Petri dishes and the fungal plugs were placed 30 mm centrally away from the actinomycetes. This test was conducted to quantitatively analyse the ability of isolates to inhibit the pathogens and to select the most potent isolates as biocontrol agents.

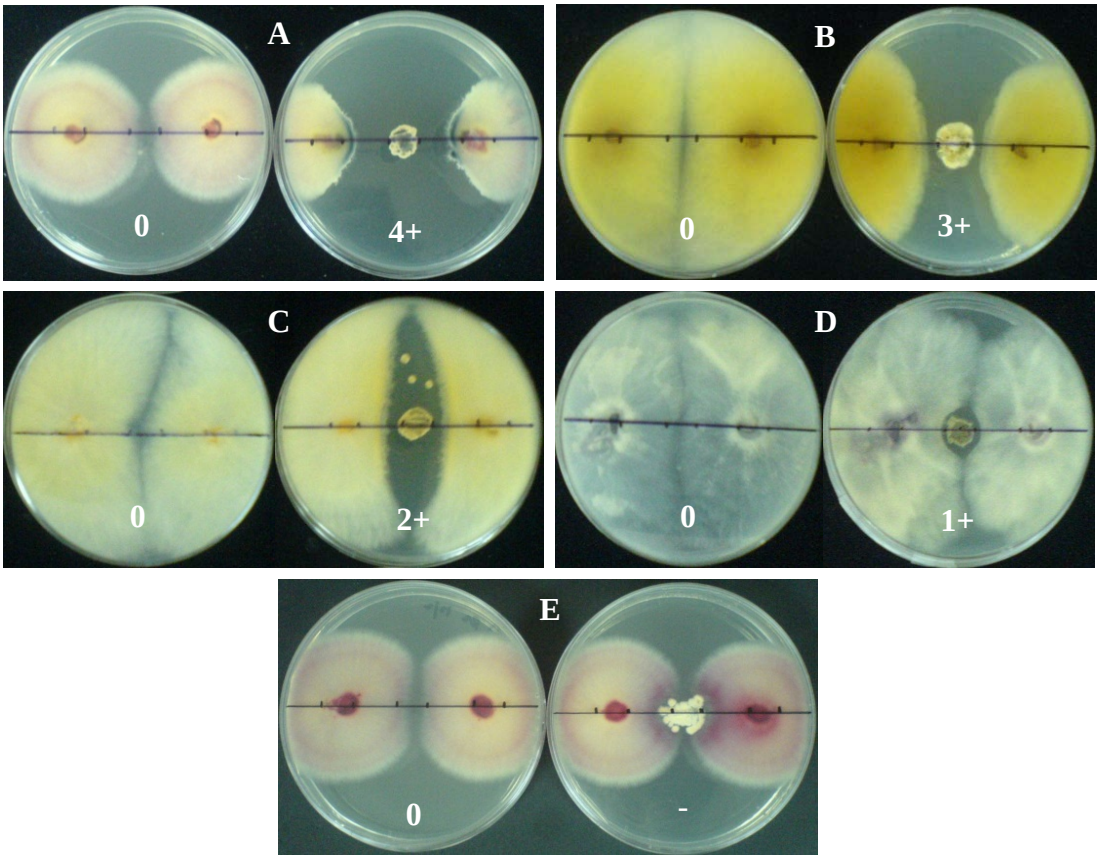

Plate 4.2: Cross-plug assay on ISP 2 agar plate after three days of incubation at 28±2°C to test the *in vitro* antagonism of actinomycetes isolates against fungi.

A & E: *Fusarium decemcellulare*

B & C: *Fusarium semitactum*

D: *Fusarium oxysporum*

(0: control, - : no inhibition, 1+: weak inhibition, 2+: moderate inhibition, 3+: strong inhibition, 4+: very strong inhibition)

Table 4.2: Evaluation of fungal growth inhibition by rhizosphere actinomycetes using the cross-plug assay method (qualitative assay).

Potential isolates	Degree of inhibition		
	R1	R2	R3
K10	2+	2+	2+
K18	3+	2+	3+
K25	1+	2+	1+
K27	2+	3+	3+
K32	2+	1+	2+
K41	3+	3+	2+
K54	2+	4+	2+
K65	2+	4+	3+
K87	2+	3+	3+
K98	2+	3+	2+
K108	1+	2+	2+
K111	1+	3+	-
K118	2+	2+	3+
K128	-	2+	2+
K136	-	-	2+
K148	2+	2+	2+
K178	1+	1+	2+
K185	3+	2+	2+
K193	3+	2+	2+
K208	2+	2+	3+
C17	3+	4+	3+
C24	2+	3+	2+
C68	1+	2+	3+

a) - : no inhibition; 1+: weak inhibition; 2+: moderate inhibition; 3+: strong inhibition; 4+: very strong inhibition

b) R1: *Fusarium oxysporum*; R2: *Fusarium decemcellulare*; R3: *Fusarium semitactum*

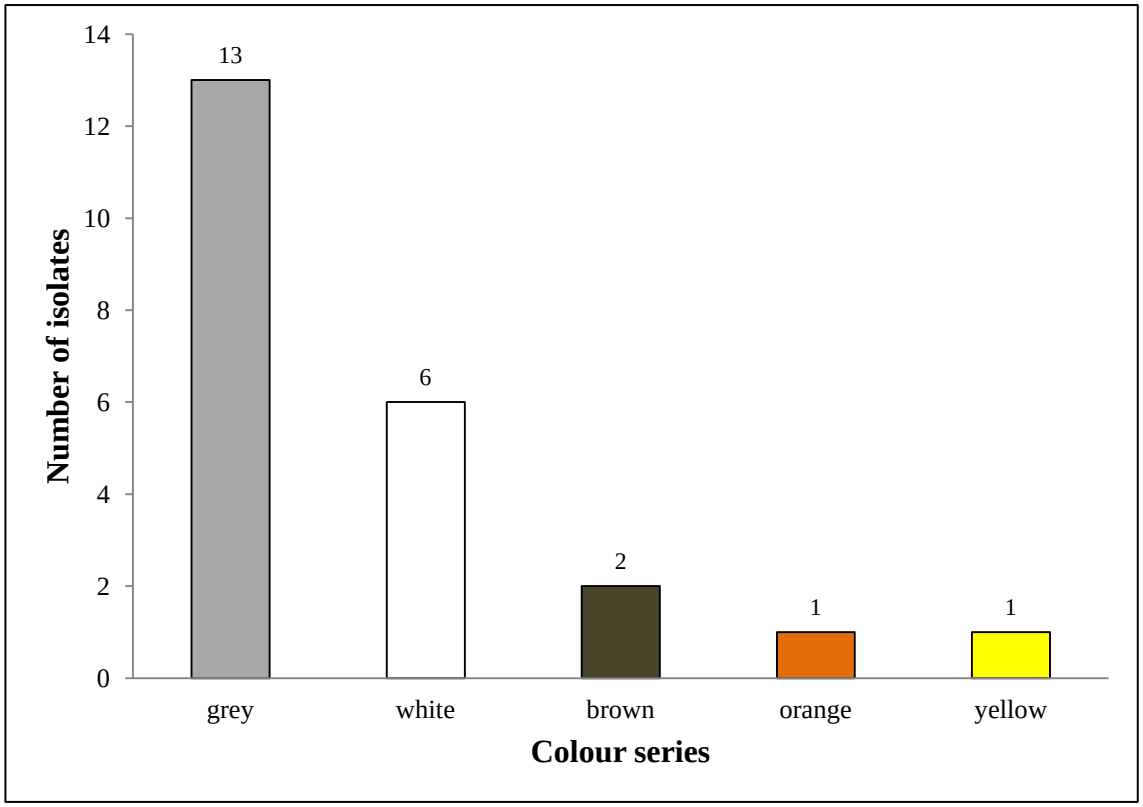

Figure 4.2: Distribution of antagonistic actinomycetes isolates based on their aerial mycelium colour in ISP 3 agar.

Figure 4.3 shows how the isolates fared in inhibiting the plant pathogens. Generally, all the isolates had some level of inhibition towards all the three fungi except for isolate K128 which was inactive towards *Fusarium semitactum* and isolate K136 which was inactive towards *Fusarium oxysporum* and *Fusarium decemcellulare*. Three isolates showed strong level of inhibition (70% to 89.3%) against all the test fungi compared to the other isolates, thus chosen for further studies. They were tentatively named as isolates C17, C68 and K98. The selected isolates belonged to the streptomycete-like group. The inhibition regions of these isolates' test plates were excised and processed for viewing through scanning electron microscope (SEM). Observations for this part will be discussed later in the chapter. Primary screening (quantitative assay) was considered sufficient in screening for antagonist as experiments carried out in this study, primarily; the inhibition mechanism, phytotoxicity and greenhouse trial were all conducted using spores, i.e., in solid media or as suspension rather than crude extracts.

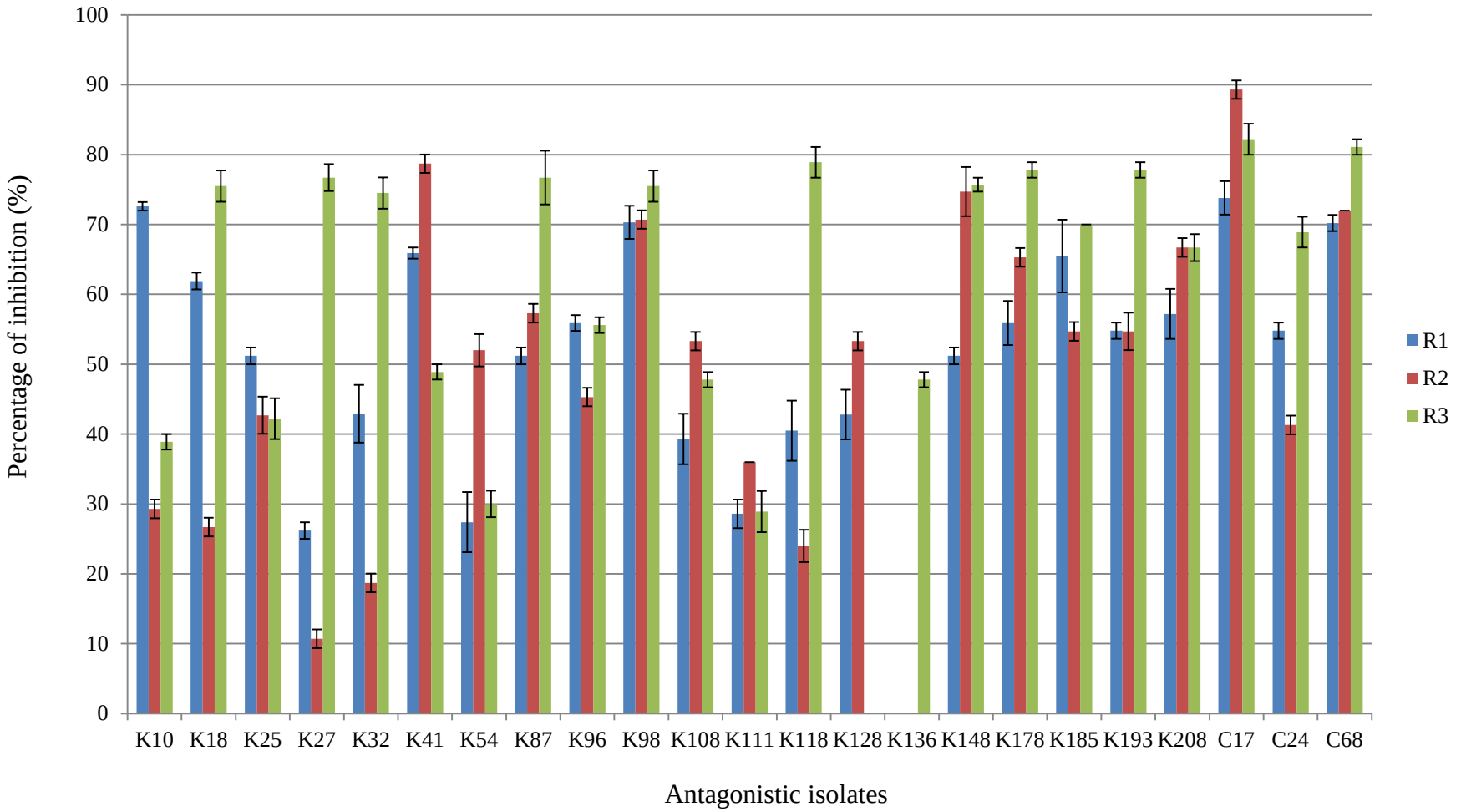

Figure 4.3: Inhibition of fungal mycelium linear growth by isolates C17, C68 and K98 (R1: *Fusarium oxysporum*; R2: *Fusarium decemcellulare*; R3: *Fusarium semitactum*; bar represents the standard error for the mean of three values). Quantitative screening was done on ISP2 plates and incubated at 28 ± 2°C for ten days.

Viability test (Yuan and Crawford, 1995) showed that, after 5 days of incubation, hyphae of test fungi taken from the periphery areas of the inhibited colonies facing isolate C17 could no longer be recovered on fresh PDA medium, indicating local death or non-culturability (at least prolonged inhibition of the growth of fungal hyphae) of the hyphae caused by the antifungal metabolites released by the isolate. In contrast, the periphery hyphae taken from the cultures facing isolates C68 and K98 were still culturable. This suggested that the secondary metabolites released by isolate C17 were fungicidal as opposed to the ones produced by the isolates C68 and K98 which were fungistatic.

Based on the results from Figure 4.4, it can be concluded that *Fusarium semitactum* was the most susceptible test fungi. Most of the antagonistic isolates (47.8 %) were able to inhibit *Fusarium semitactum* very strongly. *Fusarium oxysporum* was resistant towards the antagonist as none of the isolate was able to inhibit it very strongly.

4.4 Scanning electron microscopy of mechanisms of inhibition

The mechanisms of inhibition were studied via observation of the inhibited region through scanning electron microscope (SEM). In the presence of isolate C17, the hyphal growth of the fungal pathogens was inhibited in many ways, especially in the area of the plates facing directly to the actinomycetes colony (Plate 4.3). Plate 4.3A showed the regular radial growth of mycelial ends of a control culture of *Fusarium decemcellulare*. The mycelial ends of *Fusarium semitactum* in the inhibited region showed a folding back reaction when actinomycetes colony were introduced (Plate 4.3B, arrowed). Similar reaction was also shown by mycelium of *Fusarium decemcellulare*. Meanwhile, the mycelial ends of *Fusarium oxysporum* were arrested after attaining growth of a few

centimetres in the plate (Plate 4.3C, arrowed). Plate 4.3D (arrowed) showed a higher magnification of bulbous-like, thickened hyphae in the mid-region of the mycelium of *Fusarium oxysporum* whereas Plate 4.3E (arrowed) showed bulging particularly in the hyphal tip region of *Fusarium decemcellulare*. These reactions were observed in all three plant pathogens isolated.

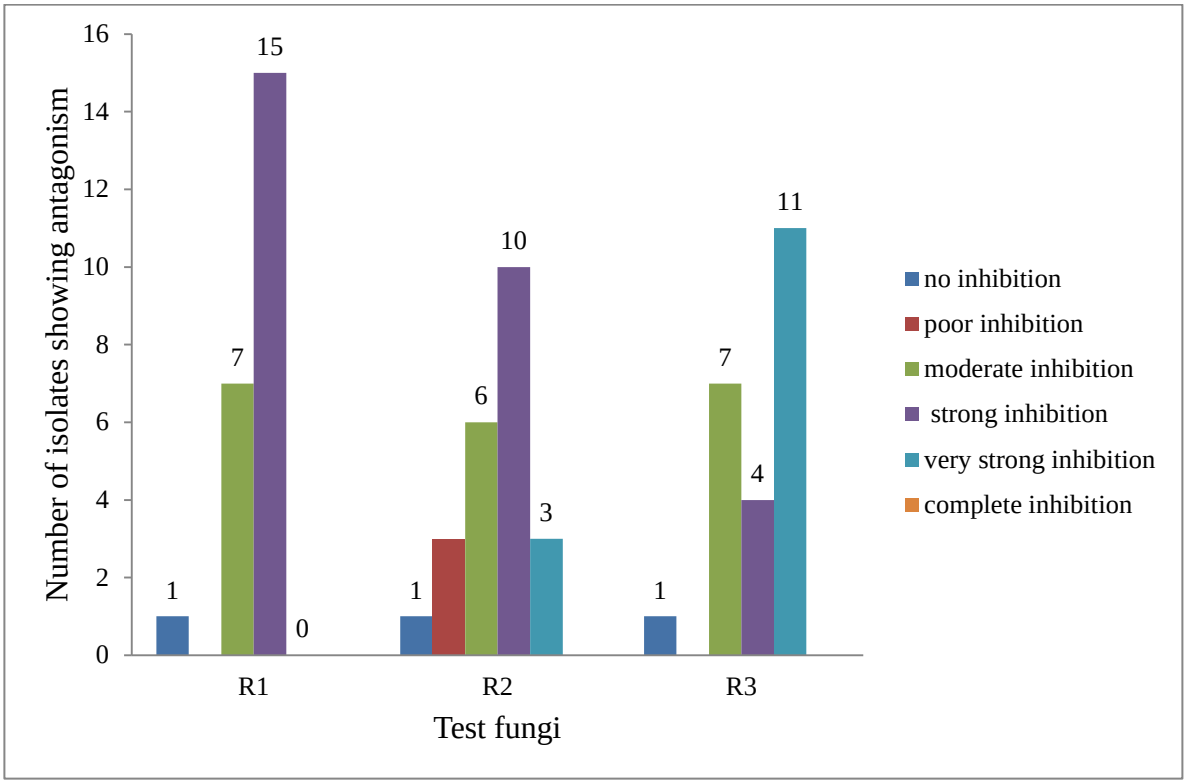

Figure 4.4: Inhibition of three different test fungi by antagonistic rhizosphere actinomycetes isolates in the agar streak screening (quantitative assay) (R1: *Fusarium oxysporum*; R2: *Fusarium decemcellulare*; R3: *Fusarium semitactum*).

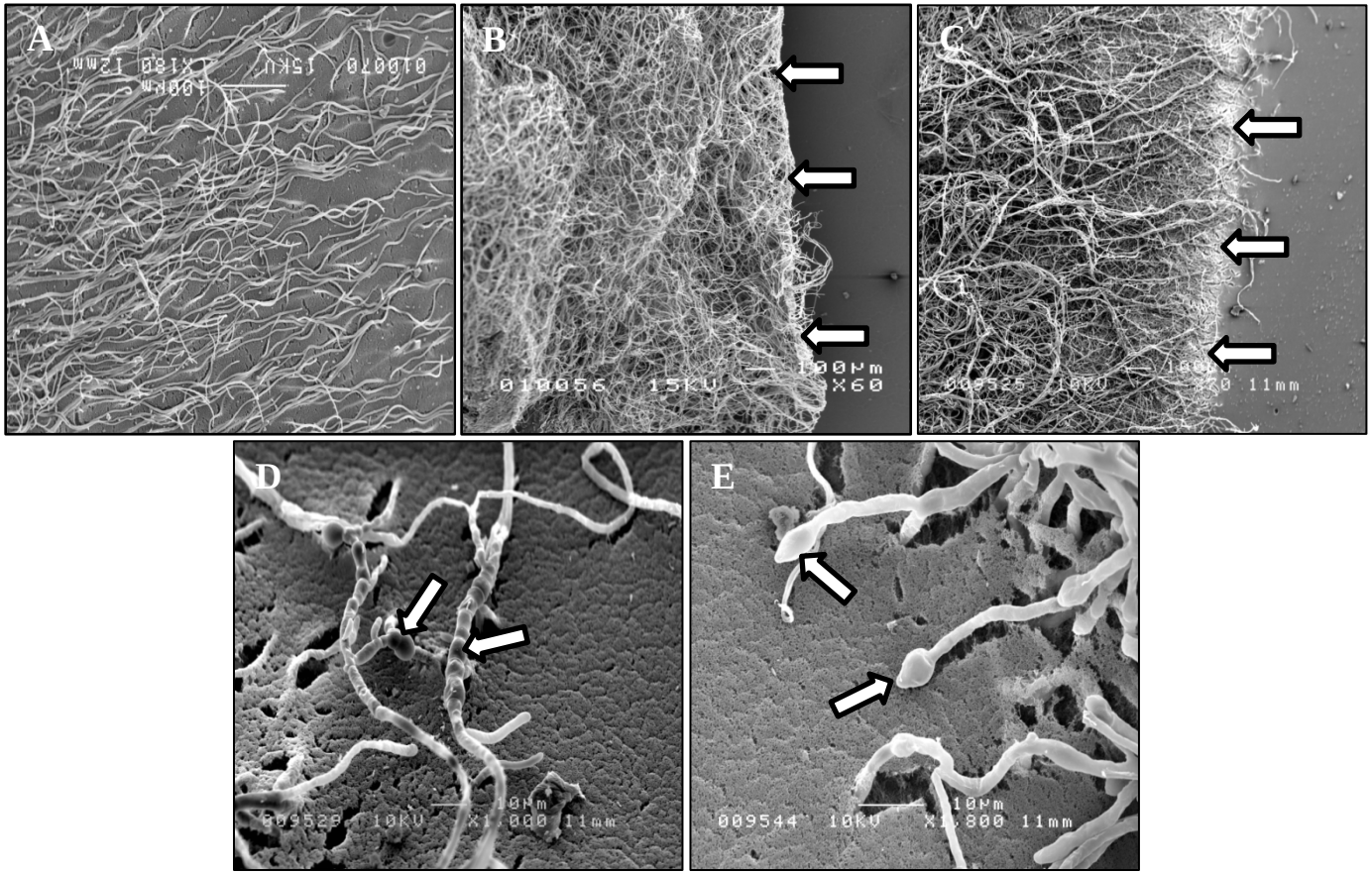

Plate 4.3: Scanning electron micrographs of hyphal ends of test fungi in the presence of isolate C17 in the agar streak assay (qualitative assay).

A: Mycelial ends of *Fusarium decemcellulare* (control plate) showing normal radial growth (bar =100µm)

B: Micrograph showing folding back of *Fusarium semitactum* hyphae (bar = 100µm)

C: Micrograph showing stunted growth of *Fusarium oxysporum* hyphae (bar = 100µm)

D: Micrograph showing swelling in the mid-region of *Fusarium oxysporum* hyphae (bar = 10µm)

E: Micrograph showing swelling in the tip of *Fusarium decemcellulare* hyphae (bar = 10µm)

For isolate C68, the hyphal growth of fungal pathogens also showed several types of inhibitions observed earlier in the presence of strain C17. Plate 4.4A showed the regular radial growth of mycelial ends of a control culture of *Fusarium oxysporum*. Mycelial ends of *Fusarium semitactum* appeared to have folded back (Plate 4.4B, red arrow) and also visible were the fragments of highly disintegrated mycelium (Plate 4.4B, yellow arrow). In Plate 4.4C, the mycelium of *Fusarium decemcellulare* appeared to form bead-like swellings (Plate 4.4C, red arrow) which eventually lead to the rupture of hyphae (Plate 4.4C, yellow arrow). Plate 4.4D shows a higher magnification of single hypha of *Fusarium oxysporum* where bulging at the tip (white arrow) and in the mid-region (black arrow) could be observed. The hyphae also appeared ruptured, splitting into two pieces with the remains clearly visible (orange arrow).

Plate 4.5A showed the regular radial growth of mycelial ends of a control culture of *Fusarium semitactum*. In the presence of isolate K98, mycelial ends of *Fusarium semitactum* appeared to form bead-like swelling with the tip looked punctured (Plate 4.5B, red arrow). In Plate 4.5C, similar symptoms were also present in the mycelium of *Fusarium decemcellulare*. Folding back of mycelium was also detected in *Fusarium decemcellulare* (Plate 4.5D). Although there was folding back of mycelium of *Fusarium oxysporum* (Plate 4.5E, white arrow), the inhibition effect seemed to be have wearied as the mycelium on the agar matrix were seen growing again (Plate 4.5E, red arrow) suggesting the inhibition effect was fungistatic.

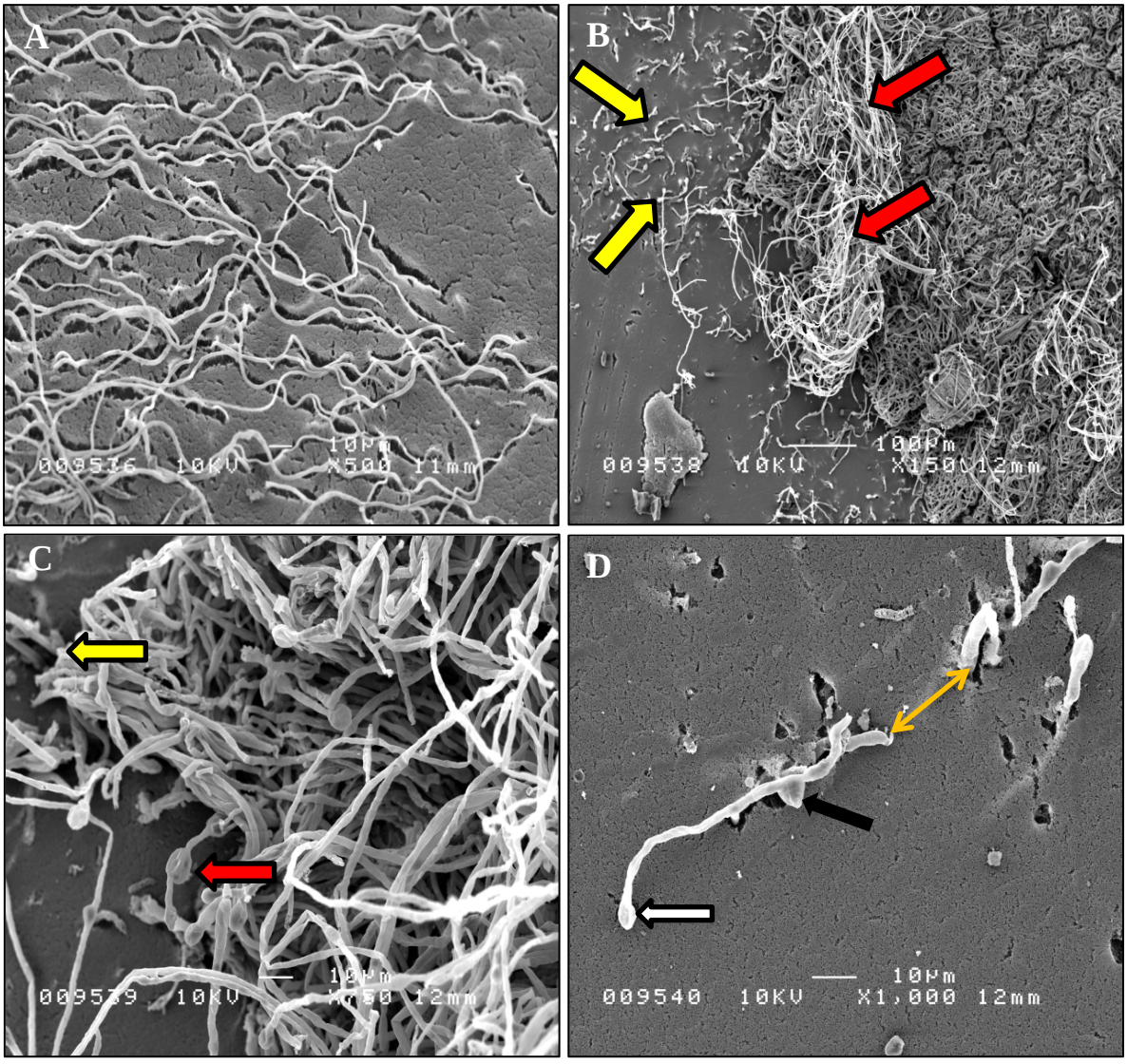

Plate 4.4: Scanning electron micrographs of hyphal ends of test fungi in the presence of isolate C68 in the agar streak assay (qualitative assay).
A: Mycelial ends of *Fusarium oxysporum* (control plate) showing normal radial growth (bar =100µm)
B: Micrograph showing folding back and disintegrated *Fusarium semitactum* hyphae (bar = 100µm)
C: Micrograph showing swelling and lysis of *Fusarium decemcellulare* hyphae (bar = 10µm)
D: Micrograph showing swelling in the mid-region and tip as well as lysis of *Fusarium oxysporum* hyphae (bar = 10µm)

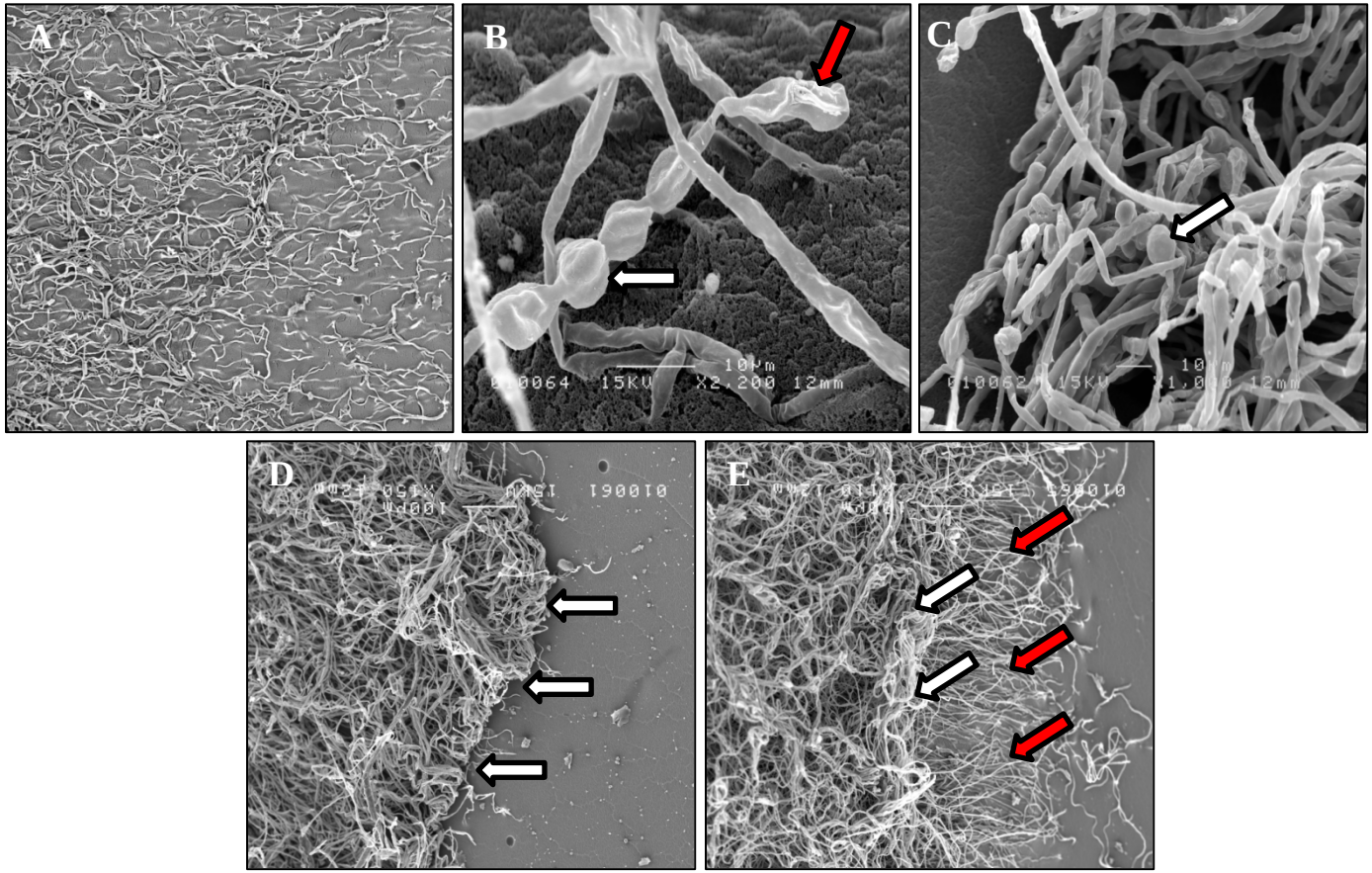

Plate 4.5: Scanning electron micrographs of hyphal ends of test fungi in the presence of isolate K98 in the agar streak assay (qualitative assay).
A: Mycelial ends of *Fusarium semitactum* (control plate) showing normal radial growth (bar =100µm)
B: Micrograph showing bead-like swelling of *Fusarium semitactum* hyphae (bar = 10µm)
C: Micrograph showing bead-like swelling of *Fusarium decemcellulare* hyphae (bar = 10µm)
D: Micrograph showing folding back of *Fusarium decemcellulare* hyphae (bar = 100µm)
E: Micrograph showing folding back of *Fusarium oxysporum* hyphae (bar = 100µm)

4.5 Micromorphological characterisation of selected antagonistic actinomycetes

The micromorphological characteristics of the three selected antagonist actinomycetes strains were studied by SEM analysis (Plate 4.6). The characterisation used in this experiment were derived from a simple classification of sporophore-spore chain types proposed by Shirling and Gottlieb (1966) which were (i) straight to flexuous, “recti-flexibilis” (RF); (ii) hooks to open loops, “retinaculum-apertum” (RA), and (iii) short to long, or compact to open spirals, “spira” (S). Of the three strains, strain C17 exhibited the S type sporophores, while strains C68 and K98 showed RF type sporohores.

Strain C17 had compact and short spirals with minimum of two turns (Plate 4.6A). The spore structure was round/barrel-like with rugose surface ornamentation (Plate 4.6B). Meanwhile, strain C68 formed a long straight chain with a minimum 10 spores (Plate 4.6C). The spores were cylindrical in shape and had smooth surfaces (Plate 4.6D). Strain K98 also formed long straight chains of spores with at least 10 spores in each chain (Plate 4.6E). The spores were also cylindrical in shape (Plate 4.6F).

4.6 Cultural studies of actinomycetes

As seen in Table 4.3 – 4.5, all three strains were able to grow well or moderately in ISP 2, ISP 3 and ISP 4 medium. Strain C17 formed grey coloured aerial spore mass on ISP 2 and ISP 4 agar media with white coloured substrate mycelium. This strain produced brown coloured soluble pigment on ISP 2, ISP 5 and ISP 7. Melanin pigment was absent on ISP 6 but present on ISP 7. Growth on ISP 2, ISP 3 and ISP 4 was good, but moderate on ISP 7 and poor on the other media tested (Table 4.3).

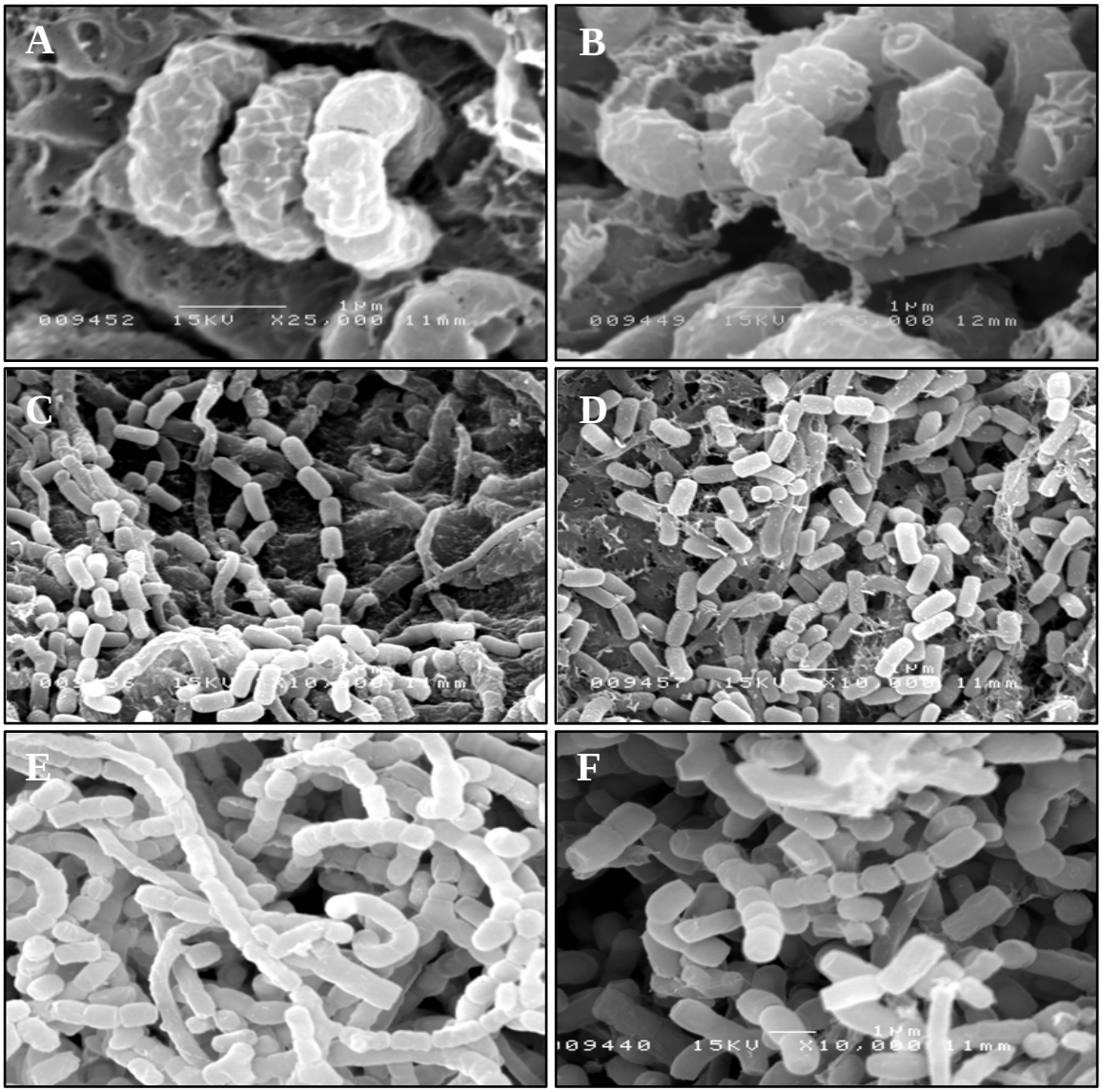

Plate 4.6: Scanning electron micrographs of spore chain types of potential actinomycetes strains C17, C68 and K98 (10 days old culture on ISP 2 agar).

A: Strain C17 (bar = 1 µm)

B: Strain C17 (bar = 1 µm)

C: Strain C68 (bar = 1 µm)

D: Strain C68 (bar = 1 µm)

E: Strain K98 (bar = 1 µm)

F: Strain K98 (bar = 1 µm)

Table 4.3: Cultural characteristics of strain C17 grown on various medium at 28±2°C for 10 days (Colour codes are indicated in parentheses below the colour designated).

Medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract-malt extract (ISP 2)	good	brownish grey (11C2)	greyish white (1B1)	dark brown (7F4)
Oatmeal (ISP 3)	good	dark grey (1F1)	grey (1C1)	none
Inorganic salts-starch (ISP 4)	good	medium grey (1E1)	white (1A1)	none
Glycerol-asparagine (ISP 5)	poor	greyish white (1B1)	light brown (7D4) with white (1A1) periphery	light brown (7D4)
Peptone-yeast extract-iron (ISP 6)	poor	none	pale yellow (1A3)	none
Tyrosine (ISP 7)	moderate	greyish white (1B1)	white (1A1)	dark brown (7F4)

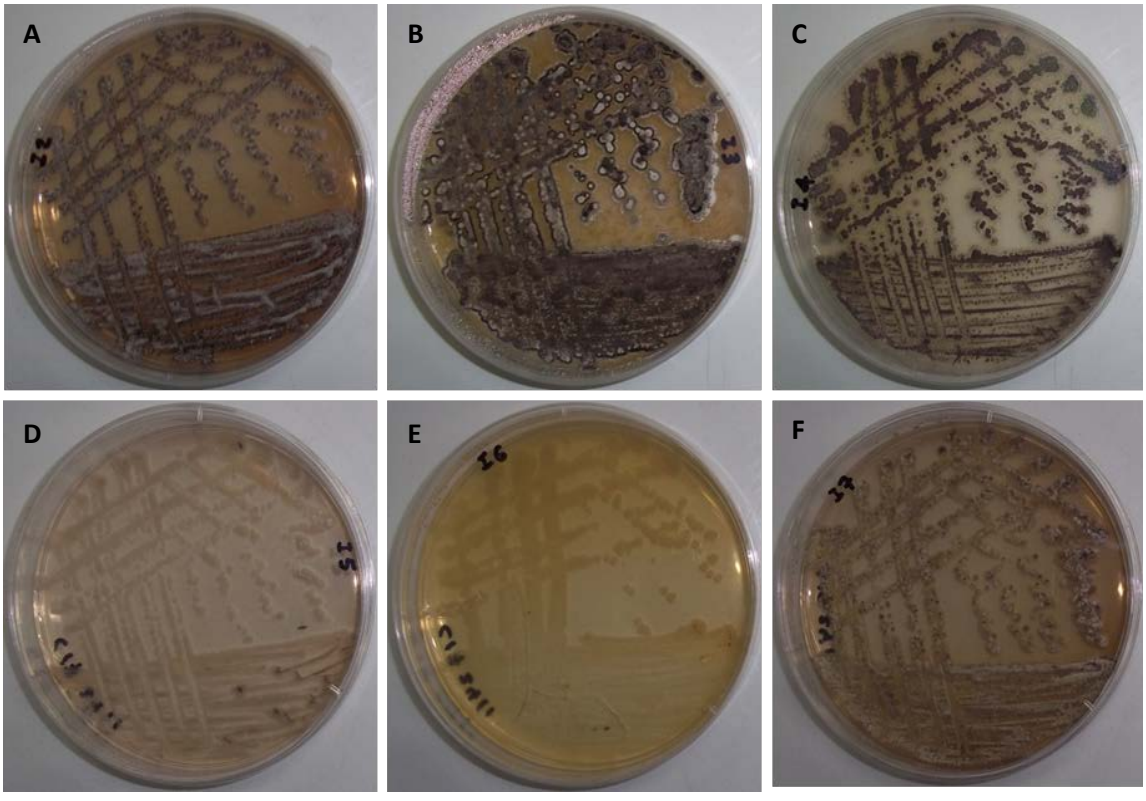

Plate 4.7: Cultural characteristics of the selected actinomycetes strain C17 after 10 days of growth at 28±2°C on various medium (A: ISP 2, B: ISP 3, C: ISP 4, D: ISP 5, E: ISP 6 and F: ISP 7)

As for strain C68, growth was good on all the media tested except ISP 3 where it was moderate. This strain produced yellow coloured aerial spore mass on ISP 2 and ISP 4, white on ISP 3, ISP 5 and ISP 6 and green in ISP 7. The substrate mycelium colour was white on all media except ISP 2. A variety of brown coloured soluble pigments were produced in ISP 2, ISP 3, ISP 6 and ISP 7. Melanin pigments were present on both ISP 6 and ISP 7 for this strain (Table 4.4).

For the K98 strain, growth was good on all media except on ISP 5 and ISP 6 where it was poor. The aerial mycelium produced was generally grey except on ISP 7 where it was white. Aerial mycelium was absent on ISP 6. Substrate mycelium was brown in colour on all medium except ISP 5 and ISP 6 where it was pale yellow. Soluble pigments were visible on ISP 4 as green and ISP 5 as brownish-yellowish. Melanin pigment was only present on ISP 7 (Table 4.5).

4.7 Other physiological characteristics of the strains

4.7.1 Degradation activity

The physiological characteristics of the selected actinomycetes are summarised in Table 4.6. Casein was degraded by strains C17 and C68 while strains C68 and K98 were able to degrade L-tyrosine. Xylan and xanthine was degraded by strains C17 and K98 respectively. All three strains were able to liquefy gelatin. Nitrate reduction activity was present in strains C17 and C68. Hydrogen sulfide production in strain C68 was confirmed by the formation of melanin pigment in ISP 6 medium.

Table 4.4: Cultural characteristics of strain C68 grown on various medium at 28±2°C for 10 days (Colour codes are indicated in parentheses below the colour designated).

Medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract-malt extract (ISP 2)	good	pale yellow (2A3)	brown (6E4)	light brown (7D4)
Oatmeal (ISP 3)	moderate	yellowish white (3A2)	white (1A1)	reddish brown (9E4)
Inorganic salts-starch (ISP 4)	good	greyish yellow (1B3)	white (1A1)	none
Glycerol-asparagine (ISP 5)	good	yellowish white (1A2)	white (1A1)	none
Peptone-yeast extract-iron (ISP 6)	good	white (1A1)	white (1A1)	black to brown (6E4)
Tyrosine (ISP 7)	good	greyish green (IC3)	white (1A1)	none

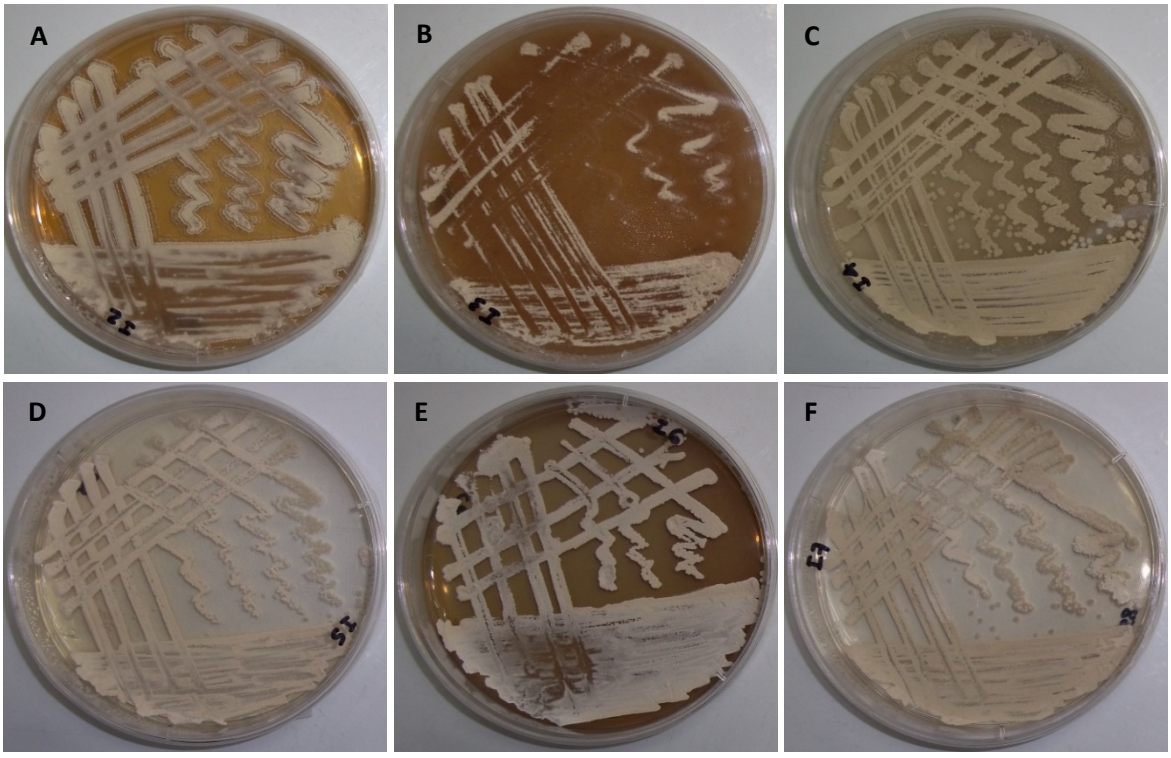

Plate 4.8: Cultural characteristics of the selected actinomycetes strain C68 after 10 days of growth at 28±2°C on various medium (A: ISP 2, B: ISP 3, C: ISP 4, D: ISP 5, E: ISP 6 and F: ISP 7).

Table 4.5: Cultural characteristics of strain K98 grown on various medium at 28±2°C for 10 days (Colour codes are indicated in parentheses below the colour designated).

Medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract-malt extract (ISP 2)	good`	greenish grey (1C2)	brown (6E4)	none
Oatmeal (ISP 3)	good	greenish grey (1C2)	brown (6E4)	none
Inorganic salts-starch (ISP 4)	good	greenish grey (1C2)	brown (6E4)	green (28B7)
Glycerol-asparagine (ISP 5)	poor	grey (1B1) with white (1A1) periphery	pale yellow (1A3)	none
Peptone-yeast extract-iron (ISP 6)	poor	none	pale yellow (1A3)	none
Tyrosine (ISP 7)	good	white (1A1)	light brown (7D4)	brown (6E4)

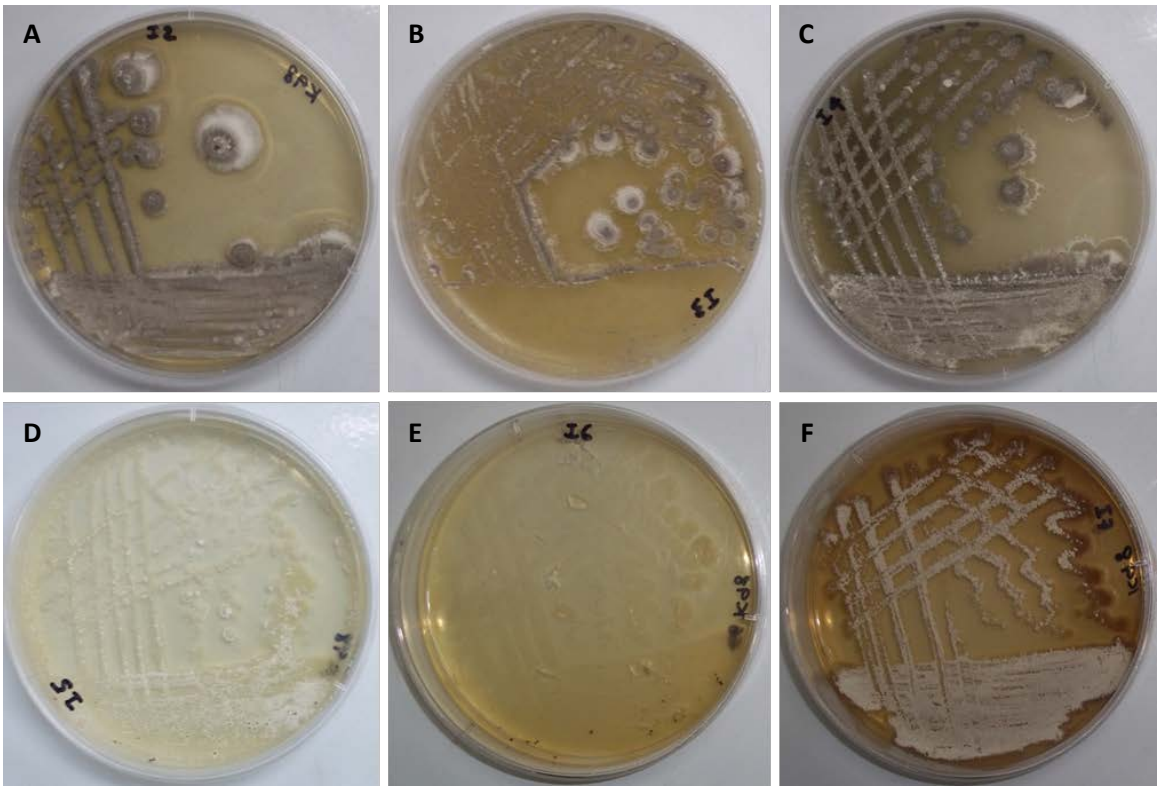

Plate 4.9: Cultural characteristics of the selected actinomycetes strain K98 after 10 days of growth at 28±2°C on various medium (A: ISP 2, B: ISP 3, C: ISP 4, D: ISP 5, E: ISP 6 and F: ISP 7).

Table 4.6: Results of other biochemical tests done for the strains.

Test	Strain		
	C17	**C68**	**K98**
Degradation			
Casein (1%, w/v)	+	+	-
Xylan (0.4%, w/v)	+	-	-
L-tyrosine (0.5%, w/v)	-	+	+
Xanthine (1%, w/v)	-	-	+
Gelatin liquefaction	+	+	+
Hydrogen sulfide production	-	+	-
Nitrate reduction	+	+	-

+, positive reaction; -, negative reaction

4.7.2 Growth at different pH, temperature and salinity levels

Generally all three strains had good growth from pH 5.5 till pH 10.5 except for strain C17 which had moderate growth at pH 10.5 and strain C68 which had moderate growth at pH 5.5 and pH 10.5 (Table 4.7).

All three strains were able to grow well at 17°C and 27°C and fail to grow at 47°C. At 7°C, strain C68 showed exceptionally good growth where the other strains failed to grow. At 37°C, the strains showed moderate to poor growth (Table 4.8).

Strain C68, although isolated from the soil, showed tolerance of up to 6% (w/v) of sodium chloride and grew poorly at 8% of sodium chloride. Strain C17 also had high salinity tolerance of up to 2% and grew moderately at 4%. Strain K98 however only grew moderately up to a salinity level of 4% and grew poorly at 6% onwards (Table 4.9).

4.7.3 Growth on sole carbon source

For the carbon utilisation test (Table 4.10), all three isolates were not able to grow or showed poor growth when cultured on ISP 9 without any supplements (basal media) which was assessed as negative control. However, for the ISP 9 media supplemented with 1% (w/v) glucose (positive control), all three strains were able to grow well.

Strain C17 generally showed good growth on ISP 9 media supplemented with L (+) -arabinose, D-mannitol, maltose, D (+)-galactose, α-L-rhamnose, β-lactose, D (-) ribose and I-inositol when compared to positive control (with D (+)-glucose). Growth on ISP 9 media supplemented with D (+)-raffinose, D-sorbitol and D (+)-mannose showed better growth than negative control but poorer than positive control. No growth was observed on sucrose and D (+)-xylose supplemented ISP 9. Strain C68 grew well on D-mannitol, D (+)-galactose, D (+)-mannose and D (-) ribose supplemented ISP 9 media. Growth on maltose was lesser compared to the positive control. This isolate was not able to utilise L (+) -arabinose, D (+)-raffinose, D-sorbitol, sucrose, α-L-rhamnose, D (+)-xylose, β-lactose and I-inositol as carbon sources on ISP 9. Strain K98 grew well on maltose, α-L-rhamnose and D (-) ribose. Growth on D (+)-galactose and D (+)-mannose was less compared to D (+)-glucose. Strain K98 was not able to grow on L (+) -arabinose, D-mannitol, D (+)-raffinose, D-sorbitol, sucrose, D (+)-xylose, β-lactose and I-inositol.

4.7.4 Susceptibility to antibiotics

All the three strains were extremely susceptible to novobiocin (Table 4.11). Strain C17 showed extreme susceptibility to all the antibiotics tested. Strain C68 was resistant towards erythromycin and was weakly susceptible towards gentamycin, neomycin and streptomycin. Fair susceptibility was observed in kanamycin and tetracycline impregnated plates. As for strain K98, it showed extreme susceptibility towards

streptomycin, kanamycin and tetracycline and was fairly susceptible against gentamycin, neomycin and erythromycin.

Table 4.7: Growth assessment of actinomycetes strains at various pH

pH	Actinomycetes strain		
	C17	C68	K98
5.5	++	+	++
6.5	++	++	++
7.5	++	++	++
8.5	++	++	++
9.5	++	++	++
10.5	+	+	++

++, good growth; +, moderate growth; ±, poor growth; -, no growth

Table 4.8: Growth assessment of actinomycetes strains at various temperatures

Temperature (°C)	Actinomycetes strain		
	C17	C68	K98
7	-	++	-
17	++	++	++
27	++	++	++
37	+	+	±
47	-	-	-

++, good growth; +, moderate growth; ±, poor growth; -, no growth

Table 4.9: Growth assessment of actinomycetes strains at various salinity levels

Salinity level (%)	Actinomycetes strain		
	C17	C68	K98
2	++	++	+
4	+	++	+
6	-	++	±
8	-	±	-
10	-	-	-
12	-	-	-

++, good growth; +, moderate growth; ±, poor growth; -, no growth

Table 4.10: Growth on sole carbon sources by the three selected strains using Pridham-Gottlieb ISP 9 agar as the basal medium.

Sole carbon source	Actinomycetes strain		
	C17	**C68**	**K98**
Basal media	-	-	-
D (+)-glucose	++	++	++
L (+) -arabinose	++	-	-
D-mannitol	++	++	-
Maltose	++	+	++
D (+)-raffinose	+	-	-
D-sorbitol	+	-	-
D (+)-galactose	++	++	+
D (+)-mannose	+	++	+
Sucrose	-	-	-
α-L-rhamnose	++	-	++
D (+)-xylose	-	-	-
β-lactose	++	-	-
D (-) ribose	++	++	++
I-inositol	++	-	-

++, growth was comparable to positive control;
+, growth was significantly better than negative control and slightly lesser than positive control;
-, no growth/poor growth compared to negative control

Table 4.11: Actinomycetes susceptibility towards selected commercial antibiotic.

Antibiotic (µg per disc)	Strain		
	C17	C68	K98
Kanamycin sulphate (30)	++ (47)	+ (23)	++ (38)
Gentamycin sulphate (10)	++ (38)	± (12)	+ (24)
Neomycin sulphate (30)	++ (37)	± (14)	+ (24)
Erythromycin (15)	++ (40)	-	+ (21)
Streptomycin sulphate (10)	++ (45)	± (19)	++ (32)
Novobiocin sulphate (30)	++ (45)	++ (36)	++ (40)
Tetracycline hydrochloride (30)	++ (40)	+ (23)	++ (37)

++: extremely susceptible (diameter inhibition zone 31- 50 mm);
+: fairly susceptible (21 - 30mm);
±: weakly susceptible (10 – 20 mm);
-: resistant
(Numbers in parentheses indicate the diameter of the inhibition zone in mm)

4.8 Phylogenetic analysis of the strains

The 16S rRNA gene sequences obtained for the three strains were compared to existing sequences in the NCBI nucleotide database using BLAST. The related sequences were then retrieved and their sequence similarity was calculated (Table 4.12). The strains were 98.3 to 100% similar to the reference sequences found in the database. Partial 16S rRNA gene sequence for each strain and the related sequences obtained from the database were aligned with CLUSTALW. The evolution history was inferred using neighbour-joining method (Saitou and Nei, 1987) as shown in Figure 4.5. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of

replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 1276 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*., 2007).

Table 4.12: Blast results of the 16S rRNA gene sequences for all strains showing the closest relatives (type strains) based on nucleotide similarity.

Strain	Sequence length	Closest relative	Identity %	Accession
C17	1364	*Streptomyces malaysiensis* strain MJM10645	100	GU350494.1
C68	1359	*Streptomyces cavourensis* subsp. *cavourensis* NBRC 13026	99.9	AB184264.1
		Streptomyces roseochromogenus NBRC 3442	99.9	AB184777.2
		Streptomyces globisporus subsp. *globisporus* NBRC 12208	99.9	AB184066.1
K98	1293	*Streptomyces olivoverticillatus* NBRC 3929	98.3	AB184812.1
		Streptomyces sanyensis strain 219820	98.3	FJ261968.2
		Streptomyces subrutilus NRRL B-12377T	98.3	DQ442545.1
		Streptomyces viridoflavus LMG20277	98.3	AJ781363.1

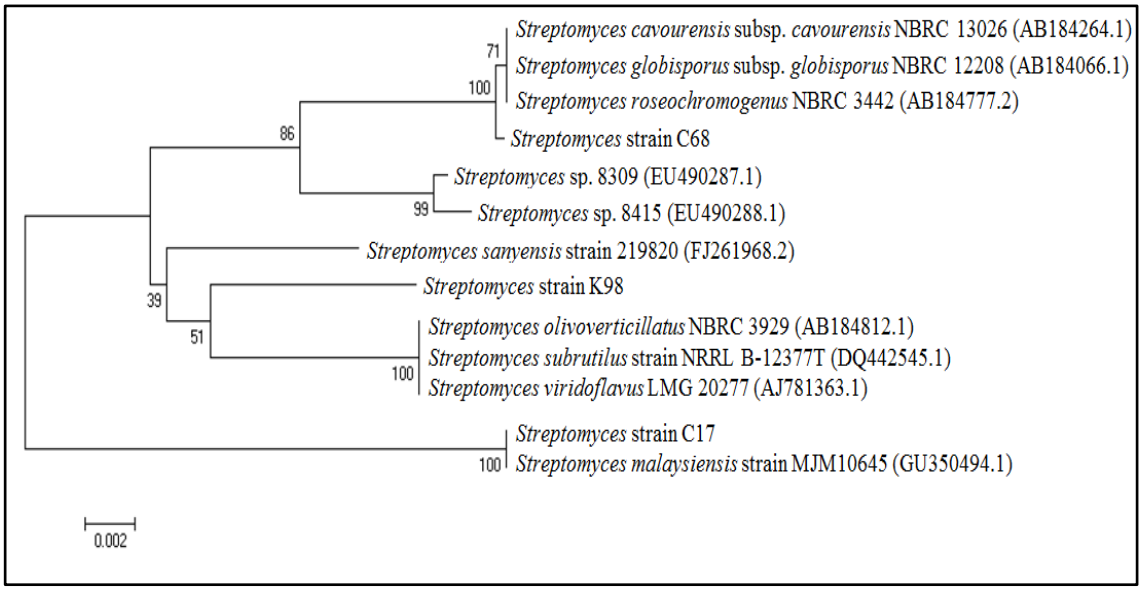

Figure 4.5: Neighbour-joining tree of the selected streptomycetes based on 16S rRNA gene sequence. The numbers at the nodes indicate the level of bootstrap support (%) based on the analysis (scale bar: substitutions per nucleotide position)

4.9 HPLC-DAD-UV-visible analysis of ethyl acetate extracts of selected *Streptomyces* strains grown on ISP 2 media

The HPLC-DAD-UV-visible analysis was conducted to identify the bioactive compounds that could be responsible for antifungal and phytotoxicity shown by the strains (Figures 4.6, 4.8 and 4.10 respectively). The bioactive compounds were identified based on the retention time and UV-visible absorbance spectra. The UV-visible absorbance spectra were later searched in an automated spectral library to classify the compounds.

The HPLC-DAD-UV-visible chromatogram of crude extract of strain C17 grown on ISP 2 medium is shown in Figure 4.6. The HPLC elution profile showed 5 major compounds. The peaks with the retention time of 9.2 min and 10.2 min had a UV spectrum identical to the geldanamycin (Figure 4.7.1 and Figure 4.7.2). Three other compounds were of bafilomycin derivatives. The peaks were obtained at retention times of 12.7 min, 14.2 min and 15.5 min. Each of them had UV spectrum identical to

bafilomycin D, bafilomycin C1 and bafilomycin B1 respectively (Figures 4.7.3, 4.7.4 and 4.7.5).

The HPLC elution profile of crude extract of strain C68 grown in ISP 2 medium produced five major compounds. The HPLC-DAD-UV-visible chromatogram for the strain is shown in Figure 4.8. The UV spectrum of compound with the lowest retention time of 6.1 min was comparable to benzoic acid (Figure 4.9.1). Two other compounds in the chromatogram were classified as maltophilin (retention time at 10.3 min – Figure 4.9.2) and dihydromaltophilin (retention time at 10.9 min – Figure 4.9.3). The final two of the five compounds were identical to the derivatives of bafilomycin. The compound identical to bafilomycin D was obtained from peak at the retention time of 12.9 min (Figure 4.9.4) whereas bafilomycin B1-like compound was obtained from peak at retention time of 15.7 min (Figure 4.9.5).

The final HPLC-DAD-UV-visible chromatogram was obtained from the crude extracts of strain K98 (Figure 4.10). This elution profile also produced five significant peaks. The first peak was at retention time of 3.4 min which produced UV-spectrum similar to 3, 5-dihydroxy-2-methyl-benzoic acid (Figure 4.11.1). The following two compounds were identical to retymicin and lagosin UV-spectrum (retention time of 6.1 min – Figure 4.11.2 and 8.5 min – Figure 4.11.3 respectively). The final two peaks were derived at retention times of 10.3 min and 10.9 min, belonging to maltophilin (Figure 4.11.4) and dihydromaltophilin (Figure 4.11.3) respectively. These two peaks were also obtained in the HLPC elution profile of strain C68.

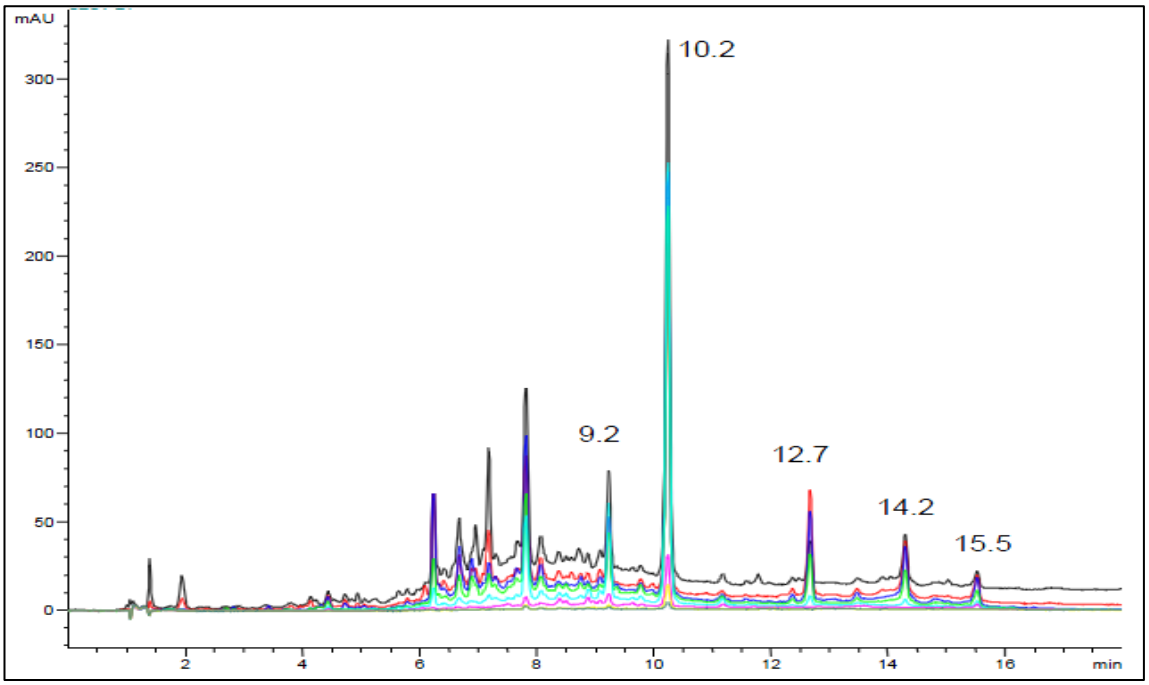

Figure 4.6: HPLC-DAD-UV-visible chromatogram of mycelial extract of *Streptomyces* sp. strain C17 on ISP 2 medium.

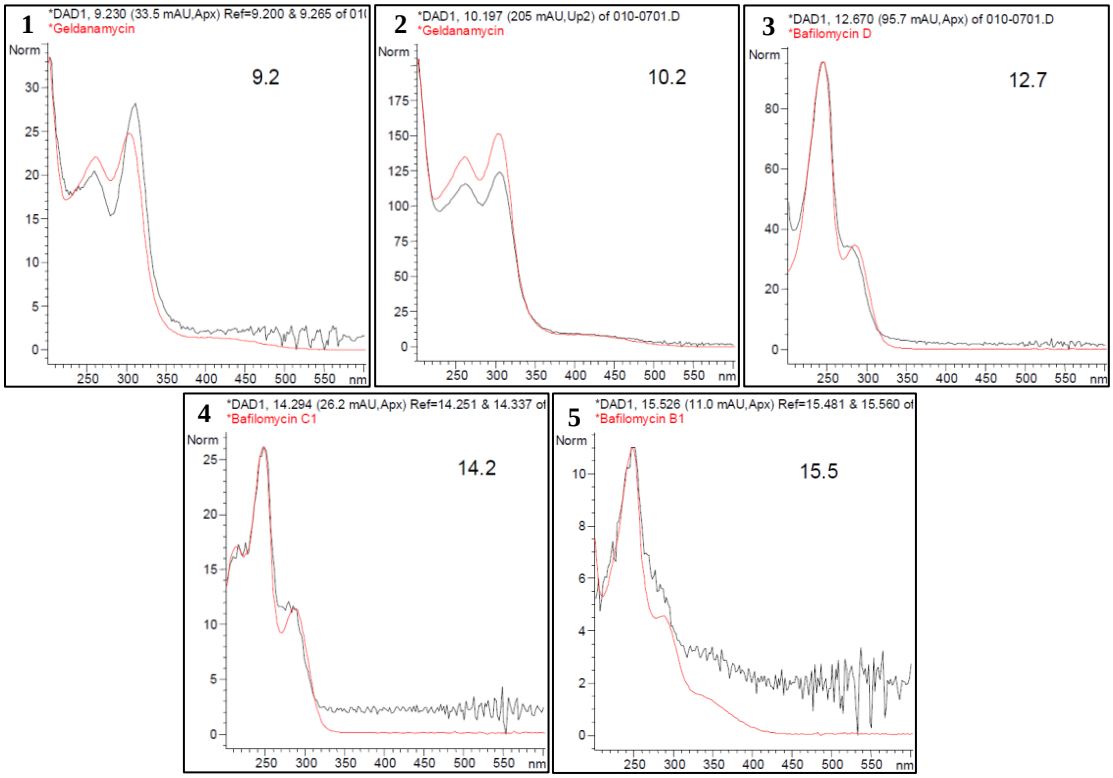

Figure 4.7: Overlaid UV-visible spectra of each peak from the mycelial extract of strain C17 (black line) and reference compound (red line).
1: Geldanamycin
2: Geldanamycin
3: Bafilomycin D
4: Bafilomycin C1
5: Bafilomycin B1

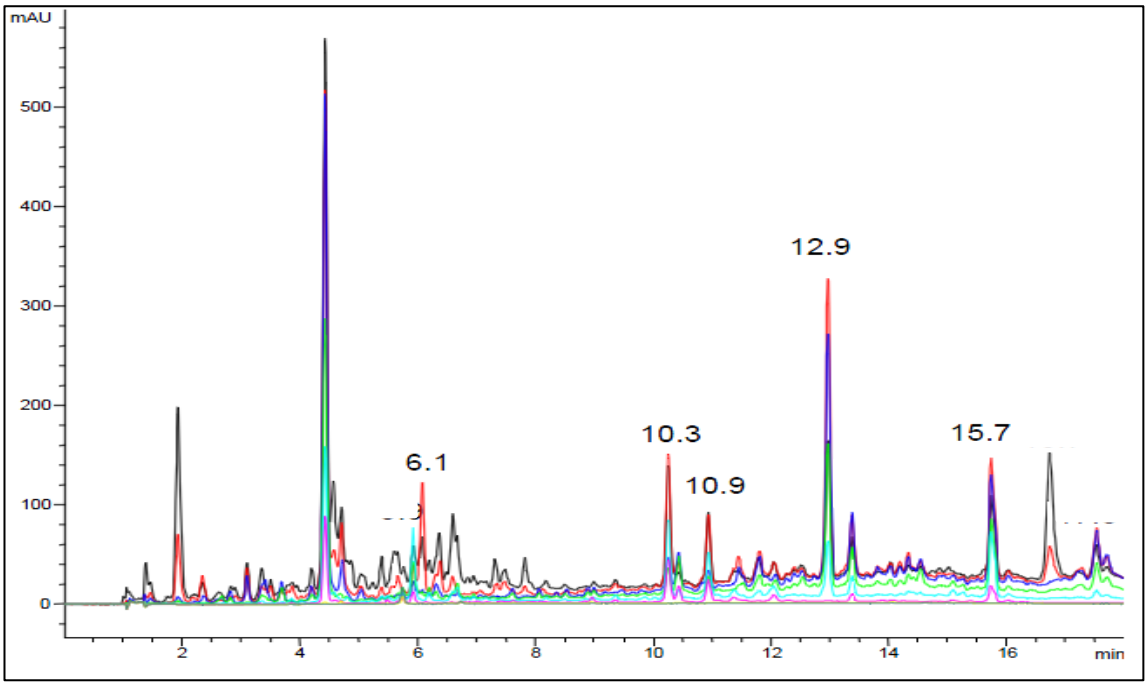

Figure 4.8: HPLC-DAD-UV-visible chromatogram of mycelial extract of *Streptomyces* sp. strain C68 on ISP 2 medium.

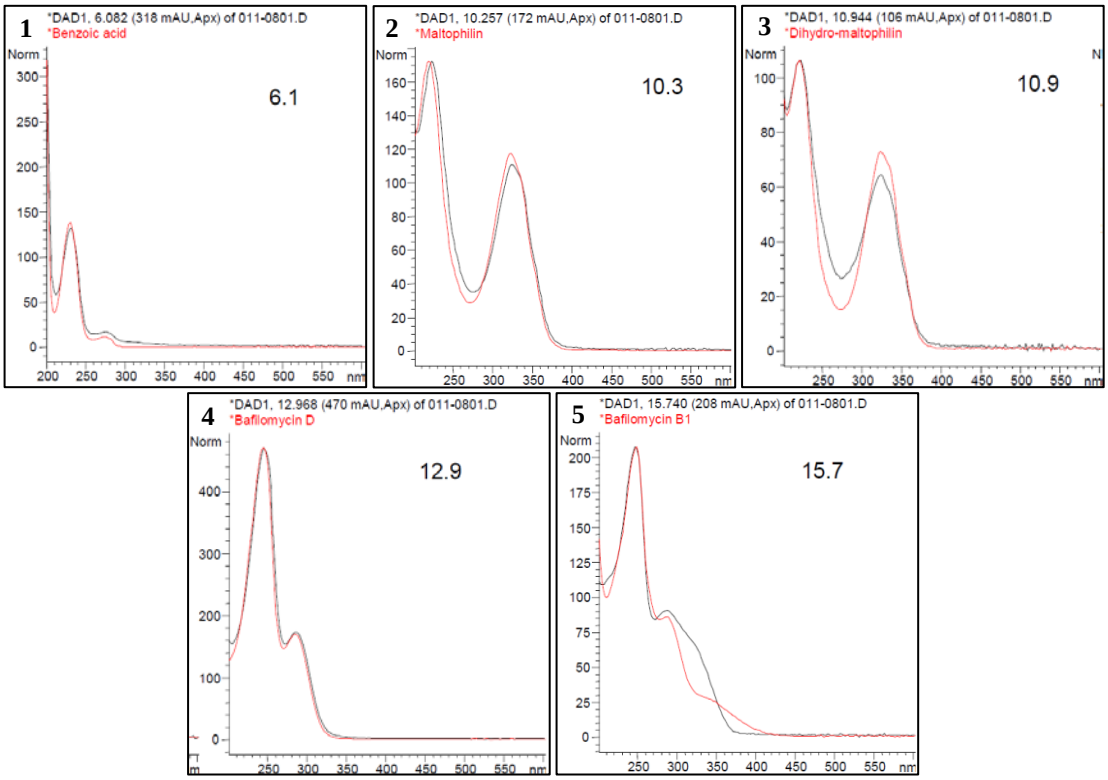

Figure 4.9: Overlaid UV-visible spectra of each peak from the mycelial extract of strain C68 (black line) and reference compound (red line).
1: Benzoic acid
2: Maltophilin
3: Dihydro-maltophilin
4: Bafilomycin D
5: Bafilomycin B1

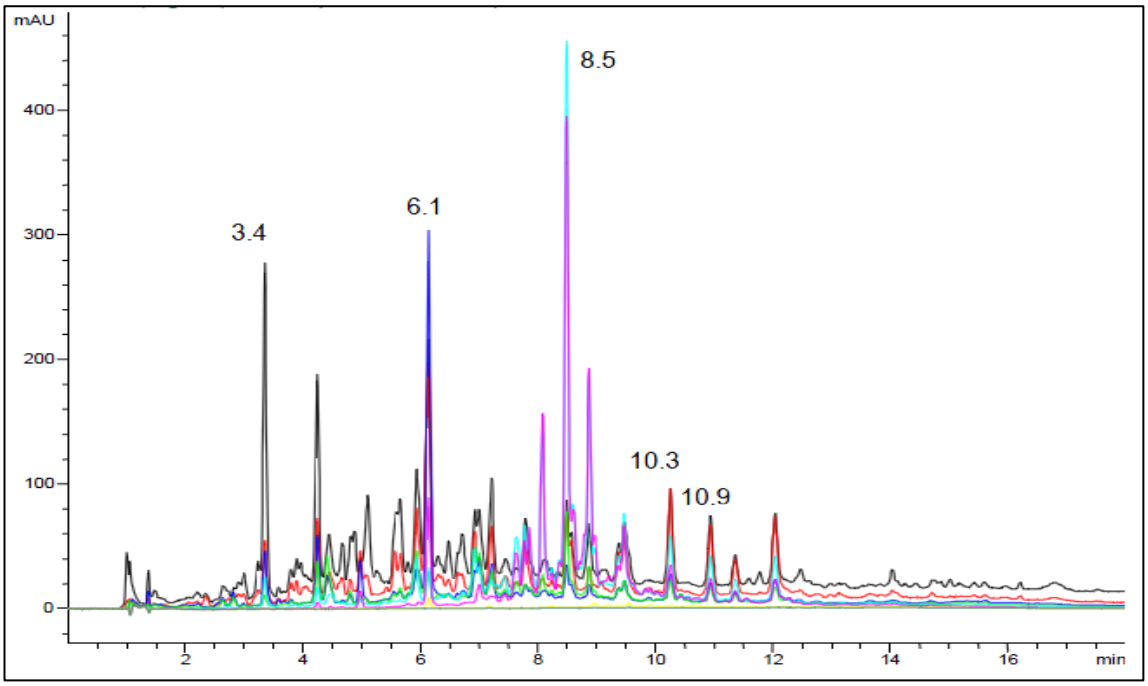

Figure 4.10: HPLC-DAD-UV-visible chromatogram of mycelial extract of *Streptomyces* sp. strain K98 on ISP 2 medium.

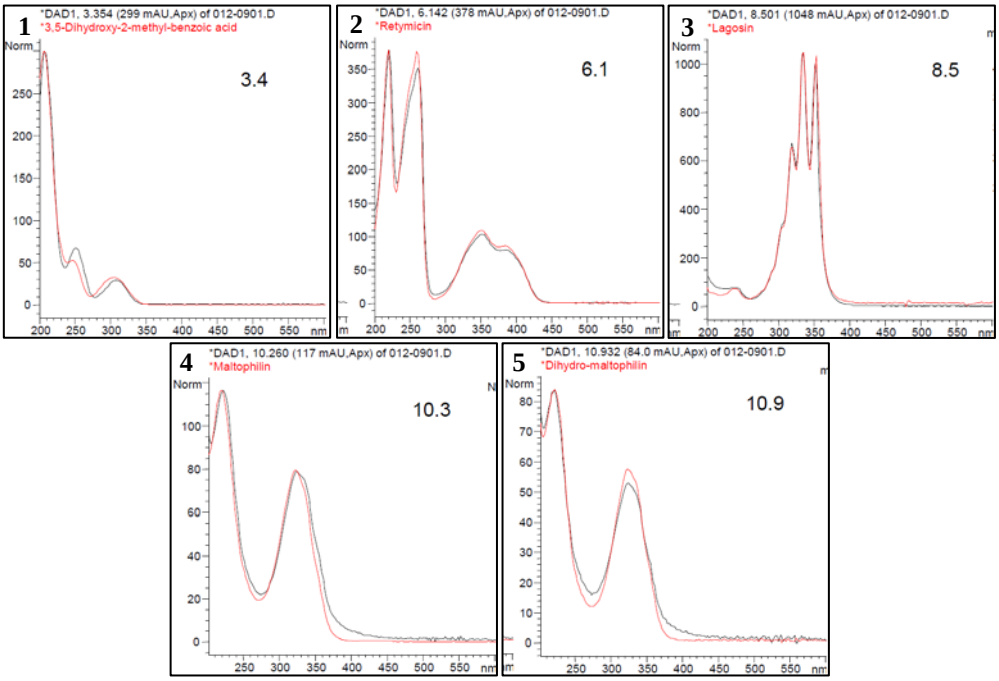

Figure 4.11: Overlaid UV-visible spectra of each peak from the mycelial extract of strain K98 (black line) and reference compound (red line).
1: 3,5-Dihydroxy-2-methyl-benzoic acid
2: Retymicin
3: Lagosin
4: Maltophilin
5: Dihydro-maltophilin

4.10 Phytotoxicity evaluation of the selected *Streptomyces* spp.

The batch of maize seeds were chosen for this assay since it could germinate consistently at average germination rate of 85.13%. The average plant height obtained from the assay was from 6.07 cm to 24.1 cm for each strain tested. On the other hand, the main root length was averaging from 5.25 cm to 33.67 cm for each strain tested. The mean for plant height and main root length for the control seedlings were 19.86 cm and 28.59 cm respectively. Duncan's multiple range tests were performed to evaluate the significant differences between the treated and control maize seedlings for the parameters evaluated.

Generally, the treatment with strains C17 and C68 reduced the height of the maize seedlings compared to the control seedlings. For the treatment with low dosage (1×10^6 CFU/ml) of strains C17 and C68, the height was reduced by 50.8% and 20.7% respectively. As for the high dosage (1×10^8 CFU/ml) treatment, the plant height was reduced by 69.4% and 18.8% respectively for strains C17 and C68. In the treatment with strain K98, the plant height was increased by 5.6% and 21.3% respectively for the low dosage treatment and high dosage treatment. All the treatments (except low dosage treatment with strain K98) were significantly ($p < 0.05$) different compared to the control seedlings.

The treatment with strains C17 and C68 also reduced the main root length of the seedlings compared to control seedlings. Low dosage treatment with strains C17 and C68 deteriorated main root length of the seedlings by 39.6% and 40.8% respectively, and it declined further in high dosage treatment by 81.6% and 67.2% respectively. However, the main root length of the seedlings was increased by 7.4% and 17.8% respectively in the low and high dosage treatment with strain K98. All the treatments (except low dosage treatment with strain K98) were significantly ($p < 0.05$) different compared to the control seedlings.

In the number of leaves produced by the maize seedlings, treatments with strains K98 and C68 improved them while the treatments with strain C17 reduced them. Treatments with strain C68 promoted the number of leaves by 7.7% in the low dosage treatment and by 3.8% in the high dosage treatment. For strain K98, the number of leaves increased by 15.4% and 19.2% respectively in the low and high dosage treatments. The high dosage treatment with strain K98 was significantly ($p < 0.05$) different compared to the control seedlings. Strain C17 reduced the number of leaves produced by 34.6% and 50% in the low and high dosage treatments. Both these treatments were significantly ($p < 0.05$) different compared to the control seedlings.

In the final characteristic observation, a different pattern was observed compared to ones earlier. Treatments with strains C17 and C68 improved the number of secondary roots. For strain C17, the number of secondary roots for the seedlings increased by 17.2% and 10.9% respectively in the low and high dosage treatments. Similarly, the number of secondary roots increased by 14.1% and 18.8% in the low and high dosage treatments with strain C68. A declining pattern was observed in the low and high dosage treatments of strain K98 where it reduced by 3.1% and 9.4% respectively. However, none of these treatments were significantly ($p < 0.05$) different compared to control seedlings.

Table 4.13: Statistical analysis of the effects of actinomycetes spore suspension treatment on the growth of maize seedlings after 10 days (low dosage: 1×10^6 CFU/ml; high dosage: 1×10^8 CFU/ml; significance value $p<0.05$, different alphabets indicate significant treatment).

Treatments	Parameter			
	Height (cm)	Main root length (cm)	Number of leaves	Number of secondary roots
Control	19.86 ± 1.77a	28.59 ± 2.33a	2.6 ± 0.52a	6.4 ± 1.65a
C17 (Low dose)	9.78 ± 1.88c	17.27 ± 2.19b	1.7 ± 0.48b	7.5 ± 2.32a
C17 (High dose)	6.07 ± 0.46d	5.25 ± 0.99c	1.3 ± 0.48b	7.1 ± 2.13a
C68 (Low dose)	15.74 ± 2.04b	16.92 ± 2.99b	2.8 ± 0.42 ac	7.3 ± 1.77a
C68 (High dose)	16.13 ± 1.97b	9.39 ± 1.84d	2.7 ± 0.48ac	7.6 ± 1.51a
K98 (Low dose)	20.98 ± 2.17a	30.71 ± 2.87a	3.0 ± 0ac	6.2 ± 1.55a
K98 (High dose)	24.1 ± 2.17e	33.67 ± 3.04e	3.1 ± 0.57c	5.8 ± 1.40a

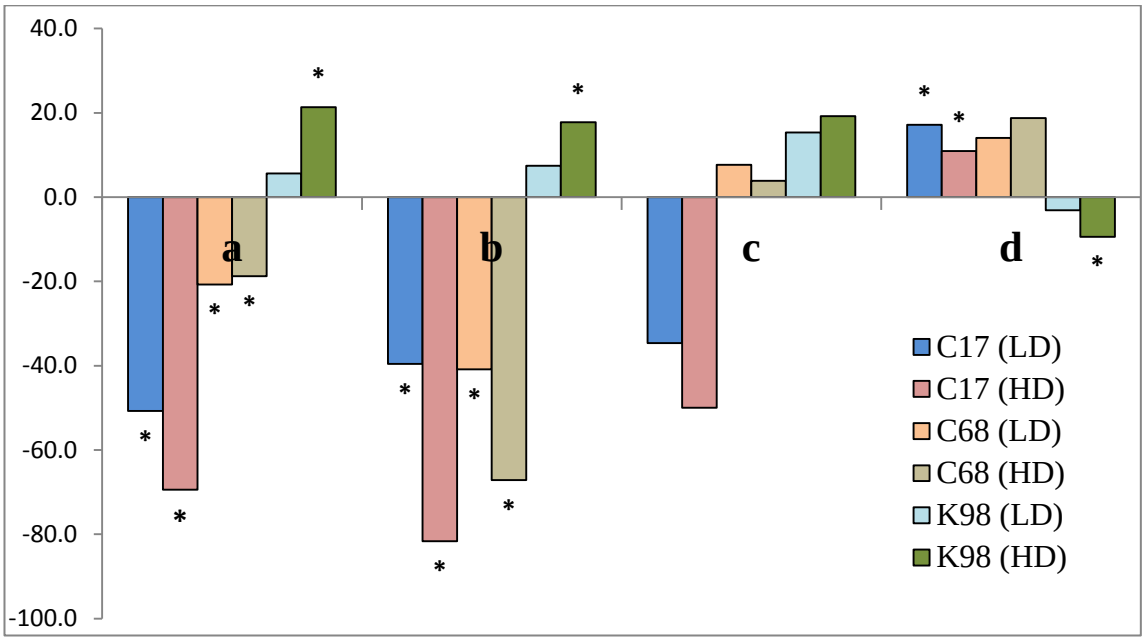

Figure 4.12: The percentage of differences in the parameters evaluated for the treated maize seedlings compared to control maize seedlings after 10 days of incubation (a: height; b: main root length; c: number of leaves; d: number of secondary roots).

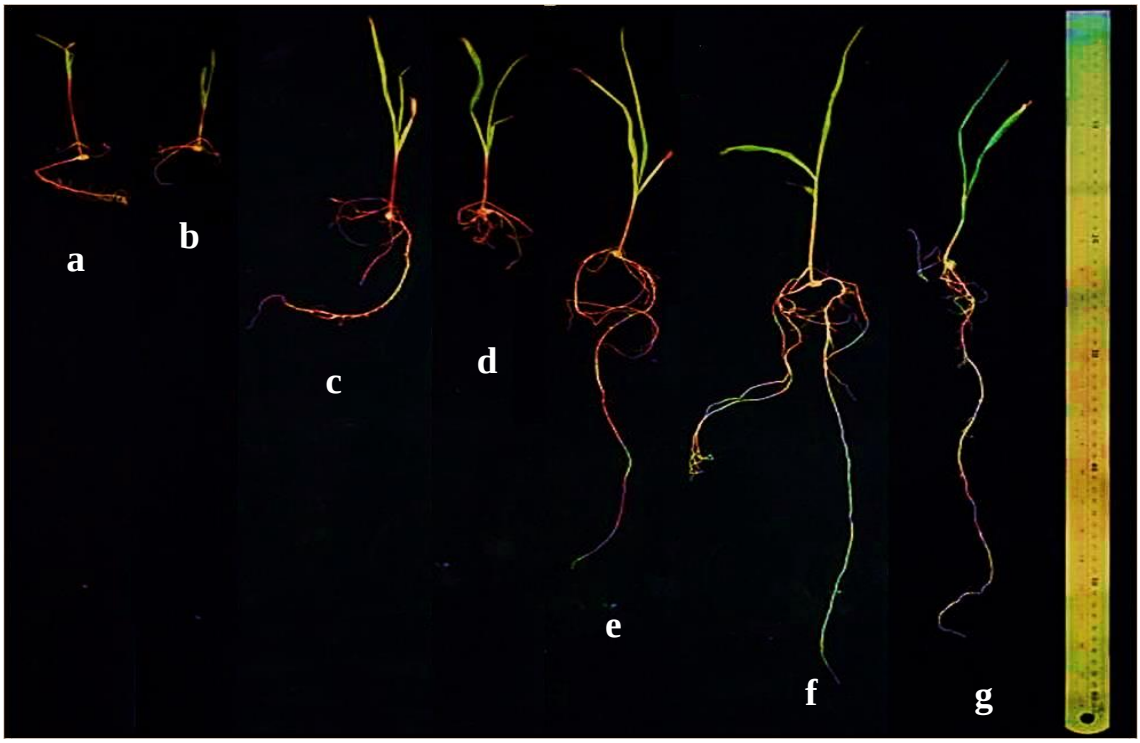

Plate 4.10: The effects of actinomycetes spore suspension on the growth of maize seedlings after 10 day (Treatments: a: C17 low dosage; b: C17 high dosage, c: C68 low dosage; d: C68 high dosage; e: K98 low dosage, f: K98 high dosage; g: control; low dosage: 1×10^6 CFU/ml; high dosage: 1×10^8 CFU/ml; Length of the ruler: 60cm).

4.11 Greenhouse trial

The spore suspension treatment selected for the greenhouse trial was the high dosage treatment (1 x 10^8 CFU/ml) based on the findings from the phytotoxicity assay. The attempt of dipping the excised region of *Hylocereus polyrhizus* stem with spore suspension of strain K98 to prevent disease infection was to no avail as all the excised stems did not show any signs of disease formation. During the whole period cultivation, no signs of major disease outbreak were observed in the control or the treated stems**.** However, there were several small disease spots observed in some of the stems from both groups. Since they did not propagate into bigger lesions, the spot formations were neglected.

There were two types of interpretation done for the parameters assessed. The first interpretation was to compare the percentage of differences between the two treatments on day 45 and day 90 for the parameters assessed as shown in Figure 4.13.

The second interpretation was to compare the percentage of differences between the same treatments from day 45 to day 90 as shown in Figure 4.14. The spore suspension dosage used for this trial was 1×10^8 CFU/ml (high dosage).

At day 45, formation of lateral shoots of the stems planted in the soil incorporated with strain K98 (hereafter will be referred to as treated stems) had reduced by 34.8% compared to the stems planted in the soil mixed with sterile distilled water (hereafter will be referred to as control stems). At day 90, the difference was reduced to 17.1%, although the formation of lateral shoots for the control stems were still higher compared to the treated stems. From day 45 to day 90, formation of lateral shoots in the treated stems proliferated by 93.3% compared to the control stems which only proliferated by 52.2%. The proliferation from day 45 to day 90 for both treated and control stems were significantly ($p < 0.05$) different.

For the total length of lateral shoots formed, the treated stems recorded reduction of 9.2% at day 45 and increased by 4.2% at day 90 compared to the control stems. Both the treated and control stems recorded a massive surge from day 45 to day 90 by 767.3% and 655.4% respectively in the total length of lateral shoots formed. The increase from day 45 to day 90 was significantly ($p < 0.05$) different for both the treated and control stems.

For the length of longest adventitious root, the treated stems were shorter by 30.7% compared to the control stems at day 45. This treatment was significantly ($p < 0.05$) different compared to the control stems. However, at day 90, the observation was reversed where the length of longest adventitious root for the treated stems were 19.7% longer than the control stems. From day 45 to day 90, the length of the longest adventitious root of the treated stems and control stems increased by 75.0% and 1.4%

respectively. The treatment with strain K98 was significantly ($p < 0.05$) different compared to the control stems.

For the total number of adventitious roots, the treated stems recorded increases of 16.5% and 5.8%, correspondingly for day 45 and day 90 compared to control stems. However, there was an unexpected decrease in the number of adventitious roots for both the treated and control stems from day 45 to day 90. The decline recorded was at 20.7% and 12.7% respectively for the treated and control stems. This could be due to errors during sampling or some of the adventitious roots could have detached while sampling or during the washing process to remove the soil particles from the roots. Alternatively, some stems might have showed delayed or late rooting which was unforeseen and thus affecting the data collected.

Table 4.14: Effects of strain K98 treatment on the formation of lateral shoots, total length of lateral shoots, length of longest adventitious root and the total number of adventitious roots after day 45 and day 90 (spore suspension dosage: 1×10^8 CFU/ml; significance value $p<0.05$, different alphabets indicate significant treatment)

Parameter	Sampling day/Treatment			
	45		90	
	SDW	K98	SDW	K98
Formation of lateral shoots	1.15 ± 0.15ab	0.75 ± 0.14a	1.75 ± 0.18c	1.45 ± 0.11bc
Total length of lateral shoots	7.81 ± 1.79a	7.09 ± 1.68a	59.00 ± 5.18b	61.49 ± 4.99b
Length of the longest adventitious root	18.49 ± 2.20b	12.82 ± 1.11a	18.75 ± 1.28b	22.44 ± 0.85b
Total number of adventitious roots	3.95 ± 0.34ab	4.60 ± 0.39b	3.45 ± 0.14a	3.65 ± 0.17a

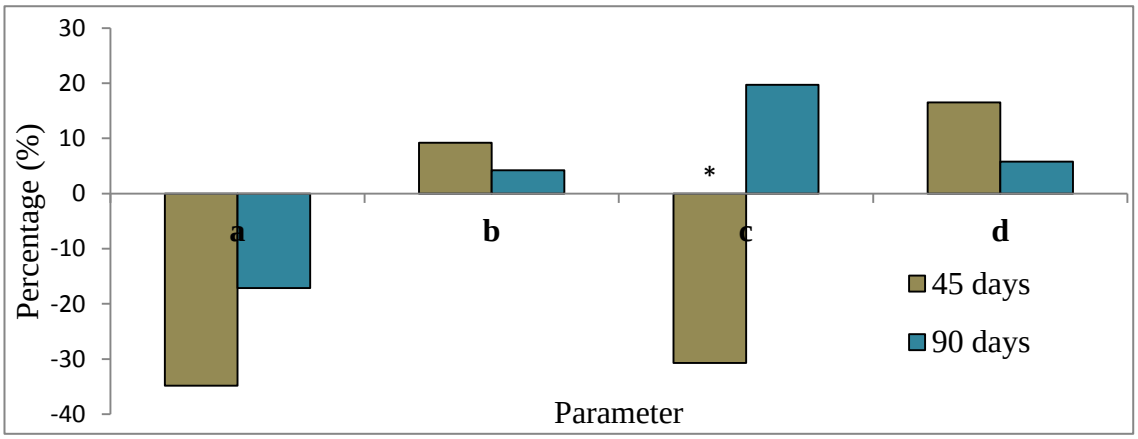

Figure 4.13: The percentage of increase or decrease in the parameters of stems treated with strain K98 compared to SDW treated stems at day 45 and day 90 (a: formation of lateral shoots; b: total length of lateral shoots; c: length of the longest adventitious root; d: total number of adventitious roots).

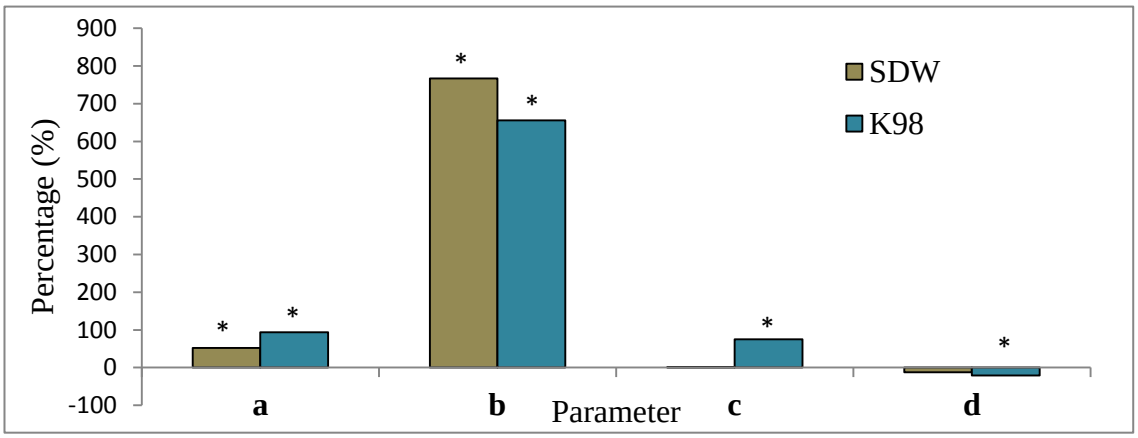

Figure 4.14: The percentage of increase or decrease from day 45 to day 90 in the parameters from the K98 treated stems and SDW treated stems (a: formation of lateral shoots; b: total length of new stems; c: length of the longest adventitious root; d: total number of adventitious roots).

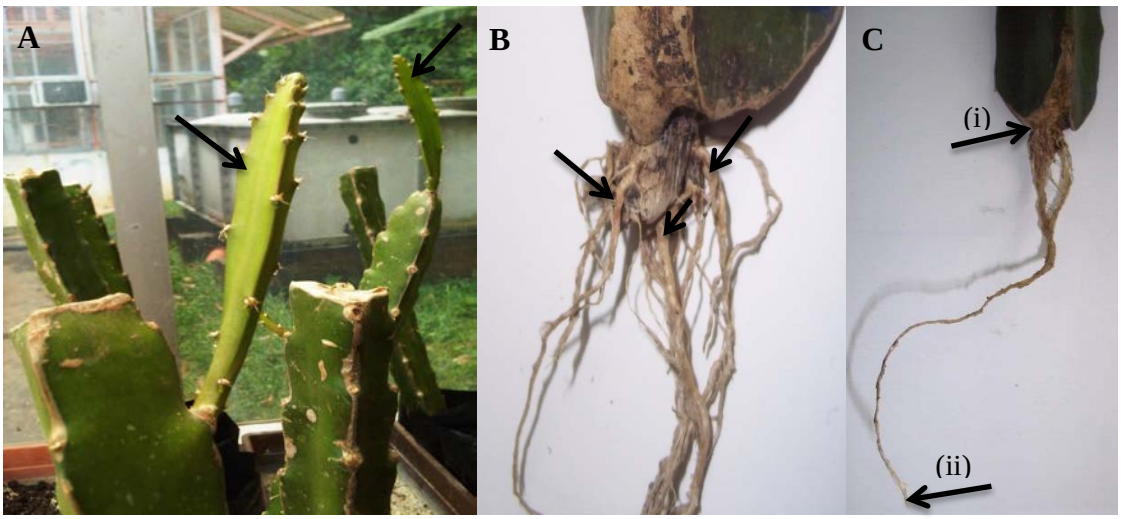

Plate 4.11: (A) Formation of lateral shoots in stems treated with strain K98 spore suspension after 45 days. (B) Adventitious roots of stems treated with strain K98 spore suspension (45 days) after washing under running water. (C) Measurements for the length longest adventitious root of the stem taken from point (i) to point (ii).

CHAPTER 5

5.0 DISCUSSION

5.1 Isolation of actinomycetes

In this study, we were interested in screening for actinomycetes from soil to function as a biocontrol agent against phytopathogens of *Hylocereus polyrhizus*. The first approach was to isolate actinomycetes from rhizosphere soil of the plant. A total of 162 actinomycetes were isolated by plating 0.1 ml of serial dilutions (10^{-2}, 10^{-3}) of moist-heated soil suspension on selective media agar, namely, starch casein agar (SCA), humic-acid vitamin agar (HVA) and raffinose-histidine agar (RHA). The media were supplemented with antibiotic such as nalidixic acid, chloramphenicol and cycloheximide to prevent bacterial and fungal contaminants from overrunning the plates. The addition of nalidixic acid was also effective in reducing bacterial growth in serial dilution method. According to Hayakawa *et al*. (1988), incorporation of these antibiotics in the selective media was not harmful to the spores of actinomycetes. Also, moist heat method was preferred in this study as Ho (2008) reported that more actinomycetes were isolated using the moist-heat method (72.44%) as opposed to dry-heat treatment (27.56%). Heat treatment of soil samples prior to isolation not only helps to reduce the numbers of Gram-negative bacteria which are commonly found in soil samples (Pisano *et al.*, 1986), it also activates spore germination of a variety of actinomycetes genera when added together with yeast extract. Furthermore, the addition of sodium dodecyl sulfate (SDS) in the pre-treatment serves a germicide to the bacterial cells.

The three media used in this study were aimed at isolating various genera of actinomycetes. Majority of the colonies that developed when SCA was used to isolate actinomycetes from soil were from *Streptomyces* spp. (Hayakawa and Nonomura,

1987). Similar observations were also obtained in the isolation of actinomycetes in mangrove rhizosphere and rhizoplane soil (Kavithambigai *et al.,* 2001). Generally, *Streptomyces* are fast growing strains and these could hinder the slow-growing actinomycetes such as *Micromonospora* and other related genera (Vikineswary *et al.,* 1997), which was why HVA and RHA were also selected as isolation media. HVA, which contains soil humic acid as the sole carbon and nitrogen source, enables the efficient recovery of actinomycetes especially the slow-growing types or rare actinomycetes such as *Micromonospora* while restricting the growth of non-filamentous bacteria (Hayakawa and Nonomura, 1987). Hayakawa and Nonomura (1987) went on to conclude that the activation of spore germination by humic acid was the reason for the increase in the diversity of actinomycetes colonies on this selective medium. On the other hand, RHA medium was originally formulated to select against strains of the frequently isolated species-group, *Streptomyces albidoflavus* and allow selective enrichment of rare streptomycete species (Vickers *et al.,* 1984). Various other pretreatment techniques can be applied to facilitate isolation of interesting actinomycetes species with special biosynthetic capabilities (Pisano *et al.,* 1986 and 1989). The addition of antibacterial and antifungal antibiotics have become an important selective technique for the isolation of different genera of actinomycetes (Labeda and Shearer, 1990) and novobiocin (Wang *et al.,* 1999) had been described for the isolation of *Micromonospora* spp. from soil. However, the use of antibiotic in this present study was focused on limiting the growth of fast-growing bacteria and fungi and not to isolate specific genera of rare actinomycetes, thus selective antibiotic were not added to the isolation media apart from the ones mentioned. For purification, the isolates were picked onto ISP 2, ISP 4 and SA. The best growth medium was determined based on the isolate's ability to show good growth. Subsequently, the same medium was maintained for that particular isolate during the course of study.

5.2 Distribution of actinomycetes isolates

The distribution of actinomycetes in this soil sample was evaluated by categorising them according to a) streptomycete-like and non-streptomycete like groups and b) aerial mycelium spore mass colour formed on ISP 3 media. Majority of the actinomycetes isolated belonged to streptomycete-like group (67.9%). Actinomycetes colonies can be recognised by their characteristics, tough, leathery or cottony texture, branched vegetative mycelia and the presence of sporulating aerial mycelium (Jensen *et al.*, 1991). The aerial mycelium colour of these isolates mainly fall into grey series (41.4%) and white series (37.7%). Other minor colour series observed were brown, yellow, green, black and orange. Each series also had different shades and some even produced soluble pigments. The range of colour of aerial mycelium and the soluble pigments produced can be an indication of the diversity and variability of *Streptomyces* in the site investigated (Ndonde and Semu, 2000; Barakate *et al.,* 2002). The differences in the color could also be due to the pigment production such as phenazines, phenoxazinones and prodiginines by the actinomycetes isolates (Rahman *et al.,* 2000).

5.3 Isolation of plant pathogenic fungi

In this study, three pathogenic fungi belonging *Fusarium* family were isolated, namely *Fusarium semitactum*, *Fusarium decemcellulare* and *Fusarium oxysporum*. Two of these strains were previously reported to be pathogenic towards *Hylocereus* spp. To our knowledge, this is the first report on the pathogenicity of *Fusarium decemcellulare* on the *Hylocereus polyrhizus*. A wide range of members from the *Fusarium* family were known to be pathogenic to *Hylocereus* spp. For example, Masratul Hawa *et al.* (2008a) reported a new disease on *H. polyrhizus* caused by *Fusarium proliferatum* which causes black to brownish lesions on stems. Besides that, *Fusarium oxysporum* were reported to cause basal rot of dragon fruit (Crane and Balerdi, 2005; Kostov and Ngan, 2006;

Wright *et al.,* 2007) as well as many other *Fusarium* species such as *F. semitectum, F. merismoides, F. compactum, F. solani, F. chlamydosporum, F. dimerum* (Hew *et al.,* 2008; Masratul Hawa *et al.,* 2008a) and *F. lateritium* (To *et al.,* 1999; Paull, 2007) which were described to be associated with diseases in dragon fruit. The discovery of *Fusarium decemcellulare* as a pathogenic agent to *H. polyrhizus*, adds to the variety of microorganisms responsible for diseases in this plant. This makes the search for biocontrol agent, as a mechanism to control the major widespread of diseases, even more important.

Fusarium decemcellulare is a common pathogen in cocoa (Ploetz, 2007) and mango (Ploetz, 2003) cultivation in the world. In Malaysia, this strain was reported to be isolated from durian, eggplant, mango, soil and vanilla. On the other hand, *Fusarium semitactum* is regularly found as a secondary invader in diseased tissues (Summerell *et al*., 2003), soils (Burgess, 1994) as well as from diverse aerial parts of plants such as maize (Andrès Ares *et al.,* 2004), asparagus (Al-Amodi, 2006), kangaroo pow (Satou *et al.,* 2001), beans (Dhingra and Muchovej, 1979), sorghum (Gopinath *et al.,* 1985), millet (Mathur *et al.,* 1975) and potatoes (Kim *et al.,* 1995). In a preliminary study conducted in 2007 on the dragon fruit diseases in Malaysia, it was revealed that the highest number of fungal isolates associated with diseased *Hylocereus polyrhizus* was *Fusarium semitectum* (Hew *et al.,* 2008; Masratul Hawa *et al.,* 2008a). *Fusarium oxysporum* is one of the most common pathogenic *Fusarium* spp. It is known to cause vascular wilts in a number of plants including banana, watermelon, roselle and long beans in Malaysia (Salleh, 2007).

5.4 *In-vitro* screening for antagonistic actinomycetes

The search for a biocontrol agent began with *in vitro* screening of actinomycetes against the fungal pathogens isolated via cross-plug assay. This assay served as a qualitative

screening as actinomycetes were first screened for inhibitory activity and level of inhibition was recorded by visual observation. By doing this, preliminary screening of large number of actinomycetes was completed in a short period of time. Selection of actinomycetes biocontrol agent by *in vitro* technique is a proper primary screening as antibiotic production is readily detectable in agar media (Omar *et al.,* 2006). Generally, isolates that exhibit non-antagonistic properties in plate's assay are also inactive in soil (Broadbent *et al.,* 1971).

Through the qualitative screening, 23% of the isolates demonstrated inhibitory activity towards at least one of the three pathogenic fungi. This is a comparatively low percentage of antagonistic isolate. This could most probably be due to the low number of test organisms used in this study. Another reason could be because the useful compounds produced by these isolates were not screened for in this study (Barakate *et al.,* 2002). Porter (1971) stated that possibly all the *Streptomyces* spp. possessed some antimicrobial properties if appropriate conditions were taken into consideration during culturing of the organisms for purposes of accessing their antibiotic production. Another possible reason could be due to the relatively low colony count of actinomycetes in the soil which can be related to the rampant spread of diseases in these plantation sites. In future, it would be more appropriate to incorporate more test fungi in the antagonism assay for a more comprehensive screening of antagonistic actinomycetes. Also, more selective isolation procedures can be added to extensive isolation to enhance the isolation of 'rare' actinomycetes, particularly species with antimicrobial capabilities.

In the quantitative screening (agar streak assay), three isolates, namely, C17, C68 and K98 showed the highest inhibitory activity (70-89%) against the fungal pathogens. It was noticeable in the quantitative screening that the agar streak assay was able to inhibit the pathogenic fungi to a greater extent than the cross-plug assay. This could be due to the fact that *Streptomyces* are known to have a relatively lower growth

rate on agar plates than most of the fungi (Yuan and Crawford, 1995). Also according to Crawford *et al.* (1993), as actinomycetes were allowed to grow and sporulate on the agar plates prior to the introduction of pathogenic fungi, the antagonism could be due to the production of secondary metabolites and most primary metabolites. Maplestone *et al.* (1992) reported that cross-correlation was observed in the genetic control mechanisms for the production of secondary metabolites and formation of spores in *Streptomyces* spp. It was noticeable that there was no physical contact between the actinomycetes and fungal hyphae, ruling out parasitism as a mechanism of antibiosis. Crawford *et al*. (1993) concluded that a large zone of inhibition on agar plates was most likely to be due to water soluble antimicrobial metabolites produced by the active isolates which may play an important role in biocontrol of plants.

The *in vitro* assay (quantitative assay) indicated that fungal phytopathogens vary in their sensitivity towards inhibition effects of actinomycetes. In this study, *Fusarium semitactum* was the most susceptible fungus as most of the antagonistic isolates (47.8 %) were able to inhibit it very strongly. *Fusarium oxysporum* was fairly resistant towards the antagonist as none of the isolates was able to inhibit it very strongly. Reddi and Rao (1971) stated that fungi that have active saprophytic life in soil were more tolerant to antagonistic actinomycetes, which was one of the factors contributing to their survival in soil.

5.5 Observation of inhibition mechanisms through scanning electron microscopy

Scanning electron microscopic was done to observe the mode of actions induced by the antagonistic actinomycetes on the hyphae of the target pathogens. The microscopic observations showed distinct morphological changes and structural alterations of the fungal mycelia compared to the control plates in the presence of actinomycetes. In this

study, fungal mycelia were found to be folding back, stunted or bulged. The bulging generally happens in the mid-region or the hyphal tip. Mycelial morphology irregularities were also observed in a study done by Patil *et al*. (2010). The mycelia of *Rhizoctonia solani* displayed reduced apical growth, curling of hyphal tips and irregular distortions in the fungal hyphae as well as pore formation on mycelial surface and cytoplasmic extrusion in the presence of a *Streptomyces* antagonist. Besides that, severe morphological changes such as excessive branching and swelling of the cell wall of *Botrytis cinerea* mycelium were observed in a co-cultured plate (Larkin and Fravel 1998; Barka *et al.,* 2002). Antibiotic substances induced malfunctions were also reported by Gunji *et al.* (1983) and Getha and Vikineswary (2002), triggering stunting, distortion, swelling, hyphal protuberances or highly branched appearance of fungal germ tubes. Disintegration of fungal cell walls would subsequently lead to cell death and to more efficient penetration by other antifungal compounds (Yuan and Crawford, 1995). It is well known that *Streptomyces* species are capable of antagonising fungal spores and hyphal structures (Yuan and Crawford, 1995), producing extracellular cell wall degrading enzymes, such as chitinase, cellulase, amylase, and 1,3-β-glucanase (Chater and Hopwood, 1989) and growth inhibiting compounds (Atta, 2009). However, there were certain streak assay plates that did not show any abnormal morphology on the fungal hyphae such as swollen or lysis of hyphal tips and abnormal branching even though inhibitions were present. Yuan and Crawford (1995) also remarked similar observations and concluded that the inhibition was most likely be due to excreted, diffusible antifungal compounds.

5.6 Identification of antagonistic strains based on genotypic and phenotypic analysis

The three strains that exhibited strong inhibitory activity (70-89%) against the fungal isolates were selected for phenotypic and genotypic characterization. Based on their 16S rRNA gene sequence similarity, the strains were 98.3 to 100% similar with the reference sequences obtained. Although 97% sequence identity value is enough to confirm species designation (Stackebrandt and Goebel, 1994), comparison of biochemical, physiological and morphological characteristics should be supplemented before the identity of the species is established.

The phylogenetic analysis revealed that strain C17 was identical to *Streptomyces malaysiensis* MJM10645 (100%) (Table 4.12; Figure 4.5). The aerial mycelium colour on ISP 4 agar was similar to the one described (Al-Tai *et al*., 1999). Compact spiral chains of cylindrical spores with rugose ornamentation observed in strain C17 were also similar to Al-Tai *et al.* (1999). Strain C17 formed melanin on ISP 7, produced diffusible pigments and hydrogen sulfide, observations which were consistent to the type strain. In the biochemical assay, degradation casein, L-tyrosine and xanthine were identical in both the isolate and the type strain. However, minor differences were visible in the carbon utilisation test, where strain C17 was able to utilise D-sorbitol and not utilise xylose, which were opposite to those observed in *Streptomyces malaysiensis*. The observation could be due to the differences in terms of habitat from which the two strains were isolated. Strain C17 was isolated from rhizosphere soil of *Hylocereus polyrhizus* farm whereas *Streptomyces malaysiensis* was isolated from soil sample collected from Tasek Bera, Malaysia (freshwater swamp lake). It is possible that D-sorbitol could be readily available from the surrounding environment of the isolation area that the isolate has adapted to utilise, as opposed to the freshwater swamp lakes where *S. malaysiensis* was isolated. The susceptibility to antibiotic test comparison

showed much discrepancy where strain C17 was found to be more susceptible to erythromycin, kanamycin and tetracycline. This could be due to environmental effects (microbial population in soil) or degeneration effects where strains tend to lose their ability after several generations of cultivation (Shirling and Gottlieb, 1977).

Strain C68 showed great 16S rRNA gene sequence similarity (99.9%) to the type strains of *Streptomyces cavourensis* subsp. *cavourensis* NBRC 13026, *Streptomyces roseochromogenus* NBRC 3442 and *Streptomyces globisporus* subsp. *globisporus* NBRC 12208 (Table 4.12). However, *Streptomyces roseochromogenus* was ruled out as possible match because of the disparity in the spore structure. Strain C68 had cylindrical spores with smooth surface ornamentation whereas *Streptomyces roseochromogenus* produce spores which were round and spiny (Shirling and Gottlieb, 1969). *Streptomyces cavourensis* subsp. *cavourensis* (Shirling and Gottlieb, 1969) and *Streptomyces globisporus* subsp. *globisporus* (Shirling and Gottlieb, 1968), both had *Rectiflexibiles* spore chain and smooth spore surface as in the current study. They also shared similarity in forming aerial mycelium of the yellow colour series in several culture media as strain C68. Carbon utilisation pattern of the two reported type strains were however different from strain C68. The two reported strains utilised arabinose and xylose as their sole carbon source unlike strain C68. This observation could be related to their respective isolation place or strain degeneration as explained earlier. The deciding factor in determining the exact match was the ability of *S. cavourensis* subsp. *cavourensis* to produce melanoid pigment on ISP 6 media and not ISP 7 media, similar to strain C68. With these comparisons, strain C68 was confidently identified as *Streptomyces cavourensis* subsp. *cavourensis* strain C68.

Strain K98 had the lowest nucleotide similarity with its closest match compared to the other strain. This could be due to the sequence length of strain K98 which was slightly shorter (62-69 base pairs) than strains C17 and C68. Strain K98 had 98.3%

similarity with *Streptomyces olivoverticillatus, Streptomyces subrutilus, Streptomycetes viridoflavus* and *Streptomyces sanyensis* strain 219820 (Table 4.12). *Streptomyces viridoflavus* was ruled out because it was reported to be a synonym of *S. olivoverticillatus. Streptomyces olivoverticillatus* was also dismissed as a possible match because it formed whirls of spore chains with spiny spores (Shirling and Gottlieb, 1969) as opposed to the spore chains of strain K98 which were straight with smooth spore surface. Comparison of aerial spore mass colour dismissed *S. subrutilus* as a match, because it produced red colour series in several culture media (Shirling and Gottlieb, 1972) whereas strain K98 formed greenish grey aerial mycelium on most of the mediums tested on. *Streptomyces sanyensis* (Sui *et al*., 2011) had a number of similar features as strain K98. The spore chain morphology and spore surface ornamentation were both similar as *recti-flexibiles* with smooth spore surface. The aerial spore mass of *S. sanyensis* was white to grey colour series and in consensus with strain K98 which had grey coloured aerial spore mass. *Streptomyces sanyensis* was positive in the tests for gelatin liquefaction, milk peptonisation and melanin production and was negative for nitrate reduction which exactly matches with strain K98. In the carbon utilization tests strain K98 and *S. sanyensis* shared the same utilisation pattern except galactose which was not utilised by *S. sanyensis.* With these comparisons, strain K98 was tentatively designated as *Streptomyces sanyensis* strain K98.

Through the identification process, it was confirmed that the antagonists belonged to the *Streptomyces* genus. This tallied with earlier observations during isolation where these isolates were placed in Streptomycete-like group due to the formation of abundant aerial spore mass in ISP 2 and ISP 4 media. It is also not surprising that the antagonists are *Streptomyces* spp. because this genus is well known for its ability to produce a variety of antifungal compounds and being the largest producer of antibiotics in the Actinomycetales family (Berdy, 2005). The genus

Streptomyces have been and remain the most fruitful source of microorganisms for agroactive metabolites (Doumbou *et al.*, 2001).

5.7 HPLC–UV–DAD analysis of actinomycetes crude extracts

Solid state fermentation was preferred in this study compared to submerged liquid fermentation. Hölker *et al*. (2004) reported that several secondary metabolites such as antibiotics and mycotoxins with significantly higher yield can be obtained in solid state fermentation compared to submerged liquid fermentation, predominantly in the cultivation of *Streptomyces,* because the production of secondary metabolites was coupled with the formation of spores by the aerial mycelia of *Streptomyces*. Hölker *et al*. (2004) cited three other works in his publication to support this. Franberg *et al.* (2000) demonstrated the production of bafilomycin B1 and C1 by *Streptomyces halstedii* k122 in solid state fermentation but not in liquid fermentation. Also, Kota and Sridhar (1999) and Yang and Ling (1989), stated that the production stability of cephamycin C by *Streptomyces clavuligerus* and tetracycline by *Streptomyces viridifaciens*, respectively, were higher in solid state fermentation than liquid fermentation.

Based on the HPLC-DAD-UV analysis, the compounds reported to be produced by the identified strains were not obtained. The compounds identified were all new to the identified strains. *Streptomyces malaysiensis* was reported to produce azalomycin F complex (Cheng *et al*., 2010) and malayamycin (Hanessian *et al*., 2003) which were not reproducible in this analysis. Similarly, *Streptomyces cavourensis* subsp. *cavourensis* was reported to produce flavensomycin (Skarbek and Brady, 1978) which was also not present in this study. Sui *et al*. (2011) reported on the activity of *Streptomyces sanyensis* against the human colon tumour cell line HCT-116 though they did not specify the secondary metabolite produced. Recently, Li *et al*. (2013) reported on the isolation and

identification of indolocarbazole (ICZ) alkaloids from *Streptomyces sanyensis* FMA. These alkaloids are interesting due to their unique structures and potential therapeutic applications. As for the compounds that were discovered in this study, a brief literature review was discussed on their origin and functions to provide a better understanding of their relatedness to this experiment.

1) Geldanamycin

Geldanamycin is an ansamycin-type antibiotic originally isolated for its antifungal property (Rothrock and Gottlieb, 1984) but was later discovered to have a potent herbicidal activity (Heisey and Putnam, 1986). This compound also interfered with RNA biosynthesis and has been reported to exhibit strong antiviral and antitumor activities (Sasaki *et al.*, 1979). It has also demonstrated moderate activity against plant pathogenic fungi from the oomycetes group (Trejo-Estrada *et al.*, 1998a).

2) Maltophilin

Maltophilin is a macrocyclic lactam antibiotic which exhibited biological activity against a broad spectrum of fungi but not sensitive towards Gram-positive and Gram-negative bacteria, and was isolated from *Stenotrophomonas maltophilia* R3089, which itself was isolated from the rhizosphere of rape plants (*Brassica napus* L.) (Jakobi *et al*., 1996).

3) Dihydromaltophilin

Dihydromaltophilin belongs to a group of tetramic acid-containing macrolactam natural products (Graupner *et al*., 1997). This heat stable antifungal factor (HSAF) metabolite is known to induce cell wall thickening which disrupts hyphal growth. Heat-stable antifungal factor (HSAF) is a secondary metabolite produced by the bacterium *Lysobacter enzymogenes* strain C3 (originally called *Stenotrophomonas maltophilia* strain C3), a biological control agent isolated from grass foliage. Strain C3 was

demonstrated in the field to reduce diseases caused by multiple fungal pathogens, including *Bipolaris sorokiniana*, *Fusarium graminearum*, *Rhizoctonia solani*, and *Uromyces appendiculatus*. It was also found to be effective in inhibiting the soilborne pathogens *Pythium ultimum* and *Magnaporthe poae* in greenhouse experiments. The mechanism by which HSAF inhibits the growth of *Aspergillus nidulans* has been studied. HSAF disrupts the polarised growth of the fungus. Genetic analysis of *A. nidulans* mutants suggests that HSAF targets the biosynthesis of sphingolipids, which are ubiquitous components of eukaryotic cell membranes and signaling molecules involved in numerous cellular processes. Interestingly, HSAF appears to target a distinct group of sphingolipids that are required for the polarised growth of filamentous fungi and appears to be absent from mammals and plants (Li *et al*., 2009).

4) Benzoic acid

Benzoic acid is an antifungal and antibacterial agent that is used as a preservative of foods (Brul and Coote, 1999). Recently, it was demonstrated that two bacteriostatic agents, enterocin and wailupemycin, both polyketides were derived from benzoic acid produced by a marine streptomycete, *Streptomyces maritimus* (Hertweck and Moore, 2000). According to Hertweck and Moore (2000), benzoic acid is also a common metabolite in eukaryotic systems and is a component of many important natural products, including salicylic acid, cocaine, taxol and the zaragozic acids. It has also been associated in plant growth inhibition and exploited after chlorination as a commercial herbicide under the name dicamba and chloramben (Bhowmik and Inderjit, 2003). Benzoic acid is reported to inhibit hydraulic conductivity and nutrient uptake by plant roots, thus resulting in growth inhibition (Blum, 1995).

5) Retymicin

Retymicin is a xanthone compound isolated from *Micromonospora* strain Tü 6368 from a soil sample collected in Romania. Retymicin show cytostatic effects to various human

tumor cell lines and Gram-positive bacteria whereas Gram-negative bacteria, such as *Escherichia coli* K12, *Pseudomonas fluorescens* DSM 50090, *Proteus mirabilis* ATCC 35501, yeasts, such as *Saccharomyces cerevisiae* ATCC 9080, *Candida albicans* Tü 164, and filamentous fungi, such as *Botrytis cinerea* Tü 157, *Aspergillus viridi nutans* CBS 12756, *Penicillium notatum* Tü 136 and *Paecilomyces variotii* Tü 137, were not sensitive against the metabolites (Antal *et al*., 2005a).

6) Bafilomycin

Bafilomycins (including A1, B and C) were isolated originally from the mycelium of *Streptomyces griseus* and were identified as macrolide antibiotics that prevented the growth of yeasts, Gram-negative bacteria and fungi (Werner *et al*., 1984) and bafilomycin D was reported to a novel insecticidal macrolides (Kretchmer *et al*., 1985) Bafilomycins has been previously reported to cause bulging of *Botrytis cinereae* hyphae (Fiedler & Fiedler, 1994). These macrolide antibiotics were produced by three *Streptomyces griseus* strains (TÜ1922, TÜ2437, and TÜ2599).

Based on the literature review above, it is safe to conclude that the production of these metabolites in the isolates lead to the inhibition of the *Fusarium* pathogens in this study. In fact, we believe that some of the mechanisms induced in the fungal mycelia during the dual culture assay could be due to reactions of these metabolites. It was observed that the presence of strains C17 and C68, which produced bafilomycins, caused bulging of the mycelia of the pathogens studied. However, it was not conclusive which compounds could have caused the other reactions or mechanisms, as no literature review were found to validate this finding. Isolation of the metabolites and testing them separately to determine the mode of action on fungal hyphae should be carried out in future studies. There were also unknown peaks which were obtained from all three strains. This could be trace compounds which are unlikely to have affected the antagonist activities of the strains. There were also several peaks unmatched with the

reference spectra in database suggesting that it could be a new secondary metabolites or derivatives of known compounds. However, no further attempt of extracting and testing them against a variety of test microorganisms to determine the nature of the secondary metabolites.

5.8 Phytotoxicity evaluation of the selected strains and greenhouse trial

Before the potential strains were tested in a greenhouse trial, they were screened for phytotoxic activity. This assay was conducted using the three selected actinomycetes strains. Maize seeds were preferred in this study compared to dragon fruit seeds because maize seeds were able to germinate much faster (after 36 hours) and showed consistent rate of germination. Dragon fruit seeds on the other hand, take at least 10 to 14 days before they germinate and the germination rates of the seedlings were inconsistent. Even in agricultural practice, *Hylocereus polyrhizus* stem cuttings were used to propagate the plants. Based on the assay, spore suspension of strains C17 and C68 were found to be toxic to the maize seedlings. Significant reduction in the height of plant and the length of main root were observed for both the low dosage (1×10^6 CFU/ml) and high dosage (1×10^8 CFU/ml) treatments. As for strain K98, significant increase was observed for the high dosage treatment in the plant height and root length of maize seedlings. To explain these observations, we have to relate to the results from the HPLC-DAD-UV analysis. Strains C17 and C68 were producing geldanamycin-like and benzoic acid-like compounds. These compounds as discussed earlier were phytotoxic. In its original study, geldanamycin inhibited radicle elongation of all the plants (barnyard grass, corn, cucumber, garden cress, large crabgrass, green foxtail, redroot pigweed, soybean, tomato and wheat) tested on a Petri dish bioassay (Heisey and Putnam, 1990). Geldanamycin also showed significant pre-emergence activity on proso millet, barnyard grass, garden cress, and giant foxtail in assays with seeds and seedlings

in field soil, but no post-emergence herbicidal effect was detected on any of the species tested. Heisey and Putnam (1990) suggested that geldanamycin is more readily taken up by roots of germinating seeds and young seedlings than foliage based on the observation that inhibitory activity was more profound in germinating seeds but lacked in post-emergence effect on larger seedlings. Therefore, geldanamycin could be more inhibitory to processes during germination or growth of emerging seedlings compared to growth of older seedlings. Herbicidal activity has also been reported for a number of metabolites produced by *Streptomyces* strains such as anisomycin and toyocamycin (Yamada *et al.*, 1972); bialaphos and glufosinate derived therefrom (Fischer and Bellus, 1983; Sekizawa and Takematsu, 1983; Seto *et al.*, 1983); cycloheximide (Ellis and McDonald, 1970; Riov and Goren, 1979; Sekizawa and Takematsu, 1983); herbicidins A and B (Arai *et al.*, 1976; Haneishi *et al.*, 1976); and herbimycins A and B (Iwai *et al.*, 1980; Omura *et al.*, 1979). More recently, Bataineh *et al*. (2005) reported the isolation of *Streptomyces* isolate R9 effectively inhibiting the germination of cucumber and ryegrass seeds and their seedling growth. The discovery of herbicidal compounds in strains C68 and C17 raises a possibility of manipulating them into formulating herbicides, even though they were initially screened as a biocontrol agent. However, more work should be done on the isolation, *in vitro* screening and field trials to validate this claim.

As for strain K98, plant growth promoting abilities were observed in both the maize seedlings and the *Hylocereus polyrhizus* stems. For the *Hylocereus polyrhizus* stems, the activity was less prevalent, where the treatment with spore suspension of K98 showed good increase in the formation of lateral shoots, total length of lateral shoots, and the length of longest adventitious root from day 45 to day 90, but less impressive when compared to the sterile distilled water (SDW) treated stems. Plant growth promoting abilities of actinomycetes have been widely studied. El-Tarabily (2008) found that *Streptomyces* spp. from tomato rhizosphere could produce IAA and promoted

tomato growth by increasing root dry weight. In the rhizosphere soils, root exudates are the natural source of tryptophan for rhizosphere microorganisms, which may enhance auxin biosynthesis in the rhizosphere. A number of mechanisms have suggested which could relate to the ability of stimulating plant growth. Some of these mechanisms have been discussed earlier in Chapter 2.

5.9 Recommendation for future studies

Future study should include more sampling sites to give a more comprehensive coverage in terms of the pathogens and antagonist isolated. The potential isolation of more fungal pathogens of *Hylocereus polyrhizus* would also lead to the screening of antagonists effective against a wide range of pathogens. In addition, the usage of selective media such as powdered chitin agar (Hsu and Lockwood, 1975) to isolate *Streptomyces* spp. which produces chitinases should also be considered. Chitinases are known to lyse fungal cell walls which contain chitin as the major component (Ueno *et al*., 1990). Furthermore, *Streptomyces* is the best studied genus for chitinase production amongst actinomycetes (Robbins *et al.*, 1988*;* Miyashita *et al.*, 1991). Doumbou *et al*. (2001) suggested that *Streptomyces* strains which produce antifungal compounds and chitinases have good potential for development into biocontrol product. As for the antagonists, more tests should be done to validate their plant growth promoting activity. For example, suitable analyses such as hormone extraction, purification and bioassay should be conducted to determine the production of plant growth regulators (PGRs) such as indole-3-acetic acid (IAA), gibberellic acid (GA3), isopentenyl adenine (iPa), isopentenyl adenoside (iPA) and zeatin (Z) in order to have a better understanding on how these antagonists are able promote plant growth. Besides that, there were peaks in the UV-visible spectra of the HPLC analysis that did not match with any of the reference compounds in the database. These compounds should be extracted and tested

for their bioactivity or sent for detailed analysis of NMR spectra to identify them as they might represent novel metabolites. Finally, to effectively measure the antagonist's ability to control disease, *in vivo* biocontrol assay should be conducted.

5.10 Conclusion

From this study, it can be concluded that 162 actinomycete isolates isolated from moist-heat treated rhizosphere soil of *Hylocereus polyrhizus.* The moist heat pre-treatment of soil samples and the addition of antibiotics in the selective media were effective in eradicating the growth of bacterial and fungal contaminants. The use of additional selective media such as humic acid-vitamin agar (HVA) and raffinose-histidine agar (RHA) to the commonly used starch-casein agar (SCA) facilitated in the recovery of actinomycetes from rare genera of actinomycetes, presumably *Micromonospora* spp. However, the exact identities of these 'rare' isolates were not examined as it was not the objective of this study. Generally, the isolates showed two significant criteria, i.e., majority of them belonged to Streptomycete-like isolates (67.9%) and the aerial mycelium colour mainly belonged to the grey series (41.4%).

The isolation of fungal pathogens from diseased *Hylocereus polyrhizus* stems resulted in the recovery of three fungi, i.e., *Fusarium semitactum, Fusarium decemcellulare* and *Fusarium oxysporum*. To our knowledge, this is the first report on the pathogenicity of *F. decemcellulare* on *H. polyrhizus*. The actinomycetes isolated were screened for their *in vitro* suppression of fungal in dual culture assay. Qualitative screening showed that 23 isolates were able to inhibit at least one pathogenic fungus. Quantitative screening indicated that isolates C17, C68 and K98 had the highest inhibitory activity (70-89%) against the phytopathogens. Phenotypic and genotypic analyses concluded that these active isolates were identified as *Streptomyces malaysiensis* (C17), *Streptomyces cavourensis* subsp. *cavourensis* (C68) and

Streptomyces sanyensis (K98). Inhibition mechanisms induced by these strains in the quantitative screening were studied via scanning electron microscopy. Distinct morphological changes of the fungal mycelia were observed in the presence of the active strains. Three morphological changes were repetitively observed on the fungal mycelia in the dual culture plates. These changes were folding back and stunted growth of mycelia and also bulging of hyphae. Based on the hyphal viability test, it was concluded that the effect of *S. malaysiensis* on the pathogens were fungicidal compared to *S. cavourensis* subsp. *cavourensis* and *S. sanyensis* which were fungistatic. The next phase of study was the identification of secondary metabolites produced by the selected strains in ISP 2 media using HPLC-DAD-UV analysis. Based on retention time and UV-visible absorbance spectra of the crude extracts, ten different compounds were identified. These compounds were geldanamycin, bafilomycins (C1, B1 and D), benzoic acid, maltophilin, dihydromaltophilin, 3,5-dihydroxy-2-methyl-benzoic acid, retimycin and lagosin. To our knowledge, the production of these compounds by *S. malaysiensis*, *S. cavourensis* subsp. *cavourensis* and *S. sanyensis* is being reported for the first time. Based on this analysis, it was concluded that the production of these metabolites lead to the inhibition activities of the *Fusarium* pathogens in the quantitative screening. Before the greenhouse trial was conducted, the selected strains were screened for phytotoxicity. *S. malaysiensis* and *S. cavourensis* subsp. *cavourensis* were found toxic to the maize seedlings in both treatments, i.e., low dosage (1×10^6 CFU/ml) and high dosage (1×10^8 CFU/ml). Significant reductions were observed in plant height and main root length of the seedlings compared to control seelings. From these observations, it was concluded that the phytotoxicity of *S. malaysiensis* and *S. cavourensis* subsp. *cavourensis* was most likely caused by the production of geldanamycin-like and benzoic acid-like compounds as shown in the HPLC-DAD-UV analysis. On the other hand, *S. sanyensis* significantly increased the plant height and main root length of maize seedlings in the

high dosage treatment (1×10^8 CFU/ml). Similar observations were also perceived in the greenhouse trial using *Hylocereus polyrhizus* stems; although the activity was less prevalent compared to sterile distilled water (SDW) treated stems. *S. sanyensis* treated (1×10^8 CFU/ml) stems were able to increase formation of new lateral shoots by 93.33% from day 45 to day 90. The *S. sanyensis* treated stems were also able to improve the length of longest adventitious root by 75.04% compared to SDW treated stems (1.41%) from day 45 to day 90.

In this study, it was demonstrated that the rhizospheric soil of *Hylocereus polyrhizus* provides a rich source of actinomycetes diversity with antifungal ability. The following information summarises the novel findings obtained from this study:

1) The isolation of *Fusarium decemcellulare* as a pathogenic fungi of *Hylocereus polyrhizus* stems;

2) The morphological changes in the fungal mycelium/hyphae caused by the introduction of *Streptomyces malaysiensis*, *Streptomyces cavourensis* subsp. *cavourensis* and *Streptomyces sanyensis* in the dual culture assay;

3) The production and characterisation of the secondary metabolites of *Streptomyces malaysiensis*, *Streptomyces cavourensis* subsp. *cavourensis* and *Streptomyces sanyensis* by HPLC-DAD-UV analysis (secondary metabolites that were not reported previously);

4) The phytotoxicity of *Streptomyces malaysiensis* and *Streptomyces cavourensis* subsp. *cavourensis* towards maize seedlings;

5) The antifungal activity and plant growth promoting ability of *Streptomyces sanyensis*.

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APPENDIX A: MEDIA

A1 Yeast extract-sodium dodecyl sulphate (SDS) solution

Yeast extract	6.0 g
SDS	0.05g
Distilled water	100 ml

(pH adjusted to 7.0 – 7.2 with 1M NaOH prior to autoclaving at 121°C for 15 min)

A2 Starch-casein agar (SCA)

Soluble starch	10.0 g
K_2HPO_4	2.0 g
KNO_3	2.0 g
Casein (vitamin free)	0.3 g
NaCI	3.0 g
$MgSO_4.7H_2O$	0.05 g
$FeSO_4.7H_2O$	0.01 g
$CaCO_3$	0.02 g
Agar	15.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 – 7.2 with 1M NaOH prior to autoclaving at 121°C for 15 min)
Antibiotics: cycloheximide (50µg/mL), nystatin (50µg/mL) and nalidixic acid (20µg/ml)

A3 Humic acid-vitamin acid (HVA)

Humic acid	1.0 g (dissolved in 10 ml of 0.2 N NaOH)
$Na_2 HPO_4$	0.5 g
KCl	1.71 g
$MgSO_4.7H_2O$	0.05 g
$FeSO_4.7H_2O$	0.01 g
Thiamin-HCl	0.5 mg
Vitamin B_2	0.5 mg
Niacin	0.5 mg
Pyridoxine-HCl	0.5 mg
Inositol	0.5 mg
Pantothenic Acid Ca-salt	0.5 mg
p-Aminobenzoic Acid	0.5 mg
Biotin	0.25 mg
Distilled water	1000 ml

(pH adjusted to 7.0 – 7.2 with 1M NaOH prior to autoclaving at 121°C for 15 min)
Antibiotics: cycloheximide (50µg/mL), nystatin (50µg/mL) and nalidixic acid (20µg/ml)

A4 Rafinose-Histidine agar (RHA)

Raffinose	10.0 g
Histidine	1.0 g
K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	0.01 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 – 7.2 with 1M NaOH prior to autoclaving)

Antibiotics: cycloheximide (50µg/mL), nystatin (50µg/mL) and nalidixic acid (20µg/ml)

A5 Yeast extract-malt extract agar (ISP 2)

Yeast extract	4.0 g
Malt extract	10.0 g
Glucose	4.0 g
NaCI	3.0 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 - 7.2 with 1M NaOH prior to autoclaving)

A6 Inorganic salts-starch agar (ISP 4)

Bacto soluble starch	10.0 g
K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	1.0 g
NaCI	10.0 g
$(NH_4)_2SO_4$	20.0 g
$CaCO_3$	2.0 g
$FeSO_4.7H_2O$	0.001 g
$MnCl_2.4H_2O$	0.001 g
$ZnSO_4.7H_2O$	0.001 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 – 7.2 with 1M NaOH prior to autoclaving)

A7 Sporulation agar (SA)

Soluble starch	20.0 g
Yeast extract	4.0 g
Agar	20.0 g

(pH adjusted to 7.0 – 7.2 with 1M NaOH prior to autoclaving)

A8 Potato dextrose agar (PDA)

PDA (Difco)	39.0 g
Distilled water	1000 ml

A9 2% Water agar (WA)

Agar (Bacto)	20.0 g
Distilled water	1000 ml

A10 Oatmeal Agar (ISP 3)

Oatmeal	20.0 g
$FeSO_4.7H_2O$	0.001 g
$MnCl_2.7H_2O$	0.001 g
$ZnSO_4.7H_2O$	0.001 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 – 7.2 with IM NaOH prior to autoclaving)

A11 Glycerol-asparagine agar (ISP 5)

Glycerol	10.0 g
L-asparagine	1.0 g

K_2HPO_4	1.0 g
$FeSO_4.7H_2O$	0.001 g
$MnCl_2 .7H_2O$	0.001 g
$ZnSO_4.7H_2O$	0.001 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 – 7.2 with IM NaOH prior to autoclaving)

A12 Peptone-yeast extract-iron agar (ISP 6)

Peptone	15.0 g
Proteose peptone	5.0 g
Yeast extract	1.0 g
K_2HPO_4	1.0 g
Ammonium Iron (II) Citrate	0.5 g
Sodium Thiosulphate	0.08 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 -7.2 with 1M NaOH prior to autoclaving)

A13 Tyrosine agar (ISP 7)

Glycerol	15.0 g
L-Tyrosine	0.5 g
L-Asparagine	1.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.5 g
NaCI	0.5 g
$FeSO_4.7H_2O$	0.001 g
MnCl2.4H2O	0 001 g
ZnSO4.7H2O	0 001 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 -7.2 with 1M NaOH prior to autoclaving)

A 14 Non-sporulating agar (NSA)

Casamino acids	20 g
Soluble starch	20 g
Yeast extract	4 g
Agar	15 g
Distilled water	1000 ml

(pH adjusted to 7.0 – 7.2 with IM NaOH prior to autoclaving)

A 15 Pridham-Gottlieb Agar medium (ISP 9)

$(HN_4)_2SO_4$	2.64 g
KH_2PO_4	2.38 g
K_2HPO_4	5.65 g
$MgSO_4.7H_2O$	1.00 g
$FeSO_4.7H_2O$	0.001 g
$MnCl_2.4H_2O$	0 0079 g
$ZnSO_4.7H_2O$	0.0015 g
$CuSO_4.5H_2O$	0.0064 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 - 7.2 with 1M NaOH prior to autoclaving)

A16 Nutrient Agar (NA)

Nutrient Agar	23.0 g
Distilled water	1000 ml

APPENDIX B: EXPERIMENTAL METHODS

B1 Scanning electron microscopy

Mycelial plugs were excised from the assay plates at designated points. Samples were fixed overnight at 2% aqueous osmium tetraxoxide at 4 °C. The fixed samples were washed with distilled water three times to remove any residues of osmium tetraoxide. Then, the mycelial plugs were dehydrated in a graded series of ethanol (15 minutes for each step) until absolute ethanol. Dehydration under absolute ethanol was done twice for 15 minutes each. The absolute alcohol was then gradually replaced by acetone (30:70, 50:50 and 70:30 parts of acetone: absolute ethanol) and finally, the materials were washed in absolute acetone twice for 15 minutes each. The dehydrated plugs in acetone were then critical point dried (C.P.D Biorad, England) using liquid carbon dioxide. The dried plugs were mounted on aluminium stubs using double sided adhesive tape, sputter-coated with gold (Biorad Cool Sputter Coater E5100) and examined in a JEOL SEM.

B2 DNA extraction

For pre-lysed sample extraction, 5 loopful of actinomycetes cell were suspended in 180 µl T1 Buffer. 25µl of Proteinase K and 5µl of 20mg/ml lysozyme were added and the mixtures were vortexed vigorously. The samples were incubated at 56°C in a hot-block, overnight, until complete lysis was obtained. The lysed samples were vortexed after the overnight incubation. Then, 200µl of B3 Buffer was added and vortexed vigorously. The mixtures were incubated at 70°C for 10 minutes and vortexed briefly after which it was centrifuged for five minutes at 13000rpm. The supernatant was then transferred to a new microcentrifuge tube. To adjust the DNA binding conditions, 210µl of ethanol (96-100%) was added to the supernatant and the mixture was vortexed vigorously. For the DNA binding process, a Nucleospin Tissue Column was placed in a collection tube and all the samples were loaded into the column and centrifuged for one minute at 13000

rpm. The flow-through was then discarded and the column was placed into the collection tube. For the washing steps, 500µl BW Buffer was added and the column was centrifuged for one minute at 13000rpm. The flow-through was discarded and the column was placed back into the collection tube. Then, 600µl B5 Buffer was added and the column was centrifuged for one minute at 13000rpm. The flow through was discarded and the column was placed back into the collection tube. The column was again centrifuged for one minute at 13000rpm to obtain dry silica membrane. To elute highly pure DNA, the NucleoSpin Tissue Column was placed into a 1.5ml microcentrifuge tube and 100µl of pre-warmed BE Buffer (70°C) was added into the column. The suspension was incubated at room temperature for about one minute. Finally, the column was centrifuged at 13000rpm for 1 minute. The eluted DNA was stored at -40°C until further use.

B3 Gel electrophoresis

Five µl DNA extracts/PCR product/purified PCR products were mixed with two µl of gel loading buffer. The mixture was loaded into the well in the agarose gel (1% w/v) in 1X SB buffer. The separation was carried out at 110 V for 30 min in SB buffer. The gel was then stained in an ethidium bromide solution and destained in distilled water. The resulting DNA patterns were examined in a UV chamber and photographed.

B4 PCR product purification using QIAquick PCR purification kit

450 µl of PBI Buffer was added to 90 µl PCR product and the suspension was mixed. The colour of the suspension was yellow, similar to PBI Buffer without the PCR sample. A QIAquick spin column was placed in a provided 2 ml collection tube. The samples were applied to the QIAquick column and centrifuged for 30 to 60 seconds at 13000 rpm to bind DNA. Then, the flow-through was discarded and the QIAquick column was placed back into the same tube. For the washing process, 0.75 ml of PE Buffer was added to the QIAquick column and the suspension was centrifuged for 30 to

60 seconds at 13000 rpm. The flow-through was then discarded and the column was placed back in the same tube. The column was centrifuged for an additional minute. The column was then placed in a clean 1.5 micro centrifuge tube. To elute DNA, 50 µl EB Buffer (10 mM Tris·Cl, pH 8.5) was added to the center of the QIAquick membrane and the column was centrifuged for one minute. The eluate was then stored at -40°C until further use.

APPENDIX C: EXPERIMENTAL AND STATISTICAL DATA

C1 Univariate ANOVA and Duncan's multiple range test (DMRT) on the effects of treatments on maize seelings in the phytotoxicity evaluation of the selected strains

C1.1 Univariate ANOVA for the effect of treatments on the height of maize seedlings

Source	Type III Sum of Squares	df	Mean Square	F	Significance
Corrected Model	2426.256[a]	6	404.376	116.294	0.000
Intercept	18131.822	1	18131.822	5214.527	0.000
Treatment	2426.256	6	404.376	116.294	0.000
Error	219.062	63	3.477		
Total	20777.140	70			
Corrected Total	2645.318	69			

[a. R Squared = 0.917 (Adjusted R Squared=0.909)]

C1.2 Summary of Duncan's multiple range test for the effect of treatments on the height of maize seedlings.[a) uses harmonic mean sample size = 10, b) alpha = 0.05 and c) error term is mean square (error) = 3.477]

Treatment	N	Subset				
		1	2	3	4	5
C17-High dose	10	6.07				
C17-Low dose	10		9.78			
C68-Low dose	10			15.74		
C68-High dose	10			16.13		
Control	10				19.86	
K98-Low dose	10				20.98	
K98-High dose	10					24.10
Sig.		1.00	1.00	0.642	0.184	1.000

C1.3 Univariate ANOVA for the effect of treatments on the main root length of the maize seedlings

Source	Type III Sum of Squares	df	Mean Square	F	Significance
Corrected Model	7219.721[a]	6	1203.287	205.345	0.000
Intercept	28724.629	1	28724.629	4901.947	0.000
Treatment	7219.721	6	1203.287	205.345	0.000
Error	369.170	63	5.860		
Total	36313.520	70			
Corrected Total	7588.891	69			

[a.R Squared = 0.951 (Adjusted R Squared = 0.947)]

C1.4 Summary of Duncan's multiple range test for the effect of treatments on the main root length of maize seedlings.[a) uses harmonic mean sample size = 10, b) alpha = 0.05 and c) error term is mean square (error) = 5.860]

Treatment	N	Subset				
		1	2	3	4	5
C17-High dose	10	5.25				
C68-High dose	10		9.39			
C68-Low dose	10			16.92		
C17-Low dose	10			17.27		
Control	10				28.59	
K98-Low dose	10				30.71	
K98-High dose	10					33.67
Sig.		1.00	1.00	0.75	0.055	1.00

C1.5 Univariate ANOVA for the effect of treatments on the number of leaves of the maize seedlings

Source	Type III Sum of Squares	df	Mean Square	F	Significance
Corrected Model	28.17	6	4.70	22.41	0.000
Intercept	422.63	1	422.63	2017.09	0.000
Treatment	28.17	6	4.70	22.41	0.000
Error	13.20	63	0.21		
Total	464	70			
Corrected Total	41.37	69			

[a.R Squared = 0.951 (Adjusted R Squared = 0.947)]

C1.6 Summary of Duncan's multiple range test for the effect of treatments on the number of leaves of maize seedlings. [a) uses harmonic mean sample size = 10, b) alpha = 0.05 and c) error term is mean square (error) = 5.86]

Treatment	N	Subset		
		1	2	3
C17-High dose	10	1.30		
C17-Low dose	10	1.70		
Control	10		2.60	
C68-High dose	10		2.70	2.70
C68-Low dose	10		2.80	2.80
K98-Low dose	10		3.00	3.00
K98-High dose	10			3.10
Sig.		1.00	1.00	0.75

C1.7 Univariate ANOVA for the effect of treatments on the number of secondary roots of the maize seedlings

Source	Type III Sum of Squares	df	Mean Square	F	Significance
Corrected Model	29.771[a]	6	4.962	1.551	0.176
Intercept	3277.729	1	3277.729	1024.799	0.000
Treatment	29.771	6	4.962	1.551	0.176
Error	201.500	63	3.198		
Total	3509.000	70			
Corrected Total	231.271	69			

[a. R Squared = 0.129 (Adjusted R Squared = 0.046)]

C1.8 Summary of Duncan's multiple range test for the effect of treatments on the number of secondary roots of maize seedlings.[a) uses harmonic mean sample size = 10, b) alpha = 0.05 and c) error term is mean square (error) = 3.477]

Treatment	N	Subset
		1
K98-High dose	10	5.8000
K98-Low dose	10	6.2000
Control	10	6.4000
C17-High dose	10	7.1000
C68-Low dose	10	7.3000
C17-Low dose	10	7.5000
C68-High dose	10	7.6000
Sig.		0.054

C2 Univariate ANOVA and Duncan's multiple range test (DMRT) on the effects of treatments/number of days on the Hylocereus polyrhizus stem in the greenhouse trial

C2.1 Univariate ANOVA for the effects of treatment/number of days on the formation of lateral shoots of the stems

Source	Type III Sum of Squares	df	Mean Square	F	Significance
Corrected Model	10.950[a]	3	3.650	8.406	0.000
Intercept	130.050	1	130.050	299.509	0.000
Treatment/days	10.950	3	3.650	8.406	0.000
Error	33.000	76	0.434		
Total	174.000	80			
Corrected Total	43.950	79			

a. R Squared = 0.113 (Adjusted R Squared = 0.078)

C2.2 Summary of Duncan's multiple range test for the effects of treatment/number of days on the formation of lateral shoots of the stems.[a) uses harmonic mean sample size = 20, b) alpha = 0.05 and c) error term is mean square (error) = 0.434]

Treatment/days	N	Subset		
		1	2	3
K98/45 days	20	0.75		
SDW/45 days	20	1.15	1.15	
K98/90 days	20		1.45	1.45
SDW/90 days	20			1.75
Sig.		0.06	0.15	0.15

C2.3 Univariate ANOVA for effects of treatment/number of days on the total length of lateral shoots of the stems

Source	Type III Sum of Squares	df	Mean Square	F	Significance
Corrected Model	55813.747[a]	3	18604.582	70.239	0.000
Intercept	91638.722	1	91638.722	345.967	0.000
Treatment/days	67.507	3	33.753	0.127	0.881
Error	20130.631	76	264.877		
Total	167583.100	80			
Corrected Total	75944.378	79			

a. R Squared = 0.735 (Adjusted R Squared = 0.724)

C2.4 Summary of Duncan's multiple range test for the effects of treatment/number of days on the total length of lateral shoots of the stems. [a) uses harmonic mean sample size = 20, b) alpha = 0.05 and c) error term is mean square (error) = 264.877]

Treatment/days	N	Subset	
		1	2
K98/45 days	20	7.0850	
SDW/45 days	20	7.8100	
SDW/90 days	20		58.9950
K98/90 days	20		61.4900
Sig.		0.888	0.629

C2.5 Univariate ANOVA for effects of treatment/number of days on length of the longest adventitious root of the stems

Source	Type III Sum of Squares	df	Mean Square	F	Significance
Corrected Model	945.920[a]	3	315.307	8.221	0.000
Intercept	26274.000	1	26274.000	685.078	0.000
Treatment/days	945.920	3	315.307	8.221	0.000
Error	2914.739	76	38.352		
Total	30134.660	80			
Corrected Total	3860.659	79			

a. R Squared = 0.245 (Adjusted R Squared = 0.215)

C2.6 Summary of Duncan's multiple range test for the effects of treatment/number of days on the length of the longest adventitious roots of the stems. [a) uses harmonic mean sample size = 20, b) alpha = 0.05 and c) error term is mean square (error) = 38.352].

Treatment/day	N	Subset	
		1	2
K98/45 days	20	12.8150	
SDW/45 days	20		18.4900
SDW/90 days	20		18.7500
K98/90 days	20		22.4350
Sig.		1.000	0.060

C2.7 Univariate ANOVA for the effects of treatment/number of days on the total number of adventitious roots of the stems

Source	Type III Sum of Squares	df	Mean Square	F	Significance
Corrected Model	15.137[a]	3	5.046	3.216	0.027
Intercept	1224.613	1	1224.613	780.466	0.000
Treatment/days	15.137	3	5.046	3.216	0.027
Error	119.250	76	1.569		
Total	1359.000	80			
Corrected Total	134.388	79			

a. R Squared = 0.113 (Adjusted R Squared = 0.078)

C2.8 Summary of Duncan's multiple range test for the effects of treatment/number of days on the total number of adventitious roots of the stems. [a) uses harmonic mean sample size = 20, b) alpha = 0.05 and c) error term is mean square (error) = 1.569].

Treatment/day	N	Subset	
		1	2
SDW/90 days	20	3.45	
K98/90 days	20	3.65	
SDW/45 days	20	3.95	3.95
K98/45 days	20		4.60
Sig.		0.239	0.105

C3 16S rRNA gene sequence of the selected actinomycetes strains

C3.1 16S rRNA gene sequence of *Streptomyces* sp. strain C17 (KM361517)

>C17
CCGGTTTCGGCCGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAAT
CTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATAT
GACGCGTTCCCGCATGGGATACGTGTGGAAAGCTCCGGCGGTGCAGGATGA
GCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGA
CGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAA
GCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACC
TCTTTCAGCAGGGAAGAAGCGTGAGTGACGGTACCTGCAGAAGAAGCGCCG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCC
GGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGATGTGA
AAGCCCGGGGCTTAACTCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTT
CGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCA
GGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAG
GAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGTTGGGAACTAGGTGTGGGCGACATTCCACGTTGTCCGTGCCGCA
GCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACT
CAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTC
GACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACATCCA
GAGATGGGTGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTC
CTGTGTTGCCAGCGGGTTATGCCGGGGACTCACAGGAGACTGCCGGGGTCA
ACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTG
GGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGAAGCCGTGAGG
TGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTC
GACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGT
GAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGG
TAACACCCGAAGCCGGTGGCCCAACCCTTGTGGAGGGA

C3.2 16S rRNA gene sequence of *Streptomyces* sp. strain C68 (KM361518)

>C68
GAAACCGCTTCGGTGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGC
AATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGA
TAATACTCCGGCCTGCATGGGTGGGGTTGAAAGCTCCGGCGGTGAAGGATG
AGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACG
ACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACG
CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAA
GCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACC
TCTTTCAGCAGGGAAGAAGCGCAAGGACGGTACCTGCAGAAGAAGCGCCG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCC
GGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGATGTGA
AGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCTAGCTAGAGTGT
GGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAG
GAGGAACACCGGTGGCGAAGGCGGACTCTGGGCCATTACTGACGCTGAGGA
GCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGT
AACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAA
AGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGAC
GCAACGCGAAGAACCTTACCAAGGTTGACATATACCGGAAAGCATCAGAGA
TGGTGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTTGTG
TTGCCAGCATGCCTTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGGGT
CAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCT
TGGGCTGCACACGTGCTACAAGGCCGGTACAATGAGCTGCGATGCCGTGAG
GCGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACT
CGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAGCATTGCGCGGT
GAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGG
TAACACCCGAAGCCGGTGGCCC

C3.3 16S rRNA gene sequence of *Streptomyces* sp. strain K98 (KM361519)

>K98
CAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGG
ATACGACCATCAGGGGCATCCTTGGTGGTGGAAAGCTCCGGCGGTGCAGGA
TGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGA
CGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCG
CAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAA
ACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGC
GCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTT
GTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGTT
GTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAG
AGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGAT
ATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGC
TGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTG
CCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTA
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTT

AATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACATCGGAAAG
TGCCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTGTTCTGTGTTGCCAGCATGCCTTTCGGGGTGATGGGGACTCACAGGAGA
CTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCC
CCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCG
ATGTCGTGAGGCGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGG
GTCTGCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAGC
ATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCA
CGAAAGTCGGTAACACC